Refine Search

Search Results -

Terms	Documents
6214344.pn.	1

Database:
US Pre-Grant Publication Full-Text Database
US Patents Full-Text Database
US OCR Full-Text Database
EPO Abstracts Database
JPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

Search: L3

Refine Search

Recall Text | Clear | Interrupt

Search History

DATE: Monday, April 02, 2007 Purge Queries Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=USPT; PLUR=YES; OP=OR			
L3	6214344.pn.	1	L3
DB=PGPB,USPT; PLUR=YES; OP=OR			
L2	Gross-milton.in.	2	L2
L1	Hay-rick.in.	2	L1

END OF SEARCH HISTORY

Refine Search

Search Results -

Terms	Documents
Gross-milton.in.	2

Database:
US Pre-Grant Publication Full-Text Database
US Patents Full-Text Database
US OCR Full-Text Database
EPO Abstracts Database
JPO Abstracts Database
Derwent World Patents Index
IBM Technical Disclosure Bulletins

Search: L2

Refine Search

Recall Text | Clear | Interrupt

Search History

DATE: Monday, April 02, 2007 **Purge Queries** Printable Copy Create Case

Set Name side by side	Query	Hit Count	Set Name result set
DB=PGPB,USPT; PLUR=YES; OP=OR			
L2	Gross-milton.in.	2	L2
L1	Hay-rick.in.	2	L1

END OF SEARCH HISTORY

```
      ---  -----  -----------
? s 2f6
      S1      53  2F6
? s met
      S2  161999  MET
? s s1 and s2
             53  S1
         161999  S2
      S3       0  S1 AND S2
? s antibod?
      S4 1718060  ANTIBOD?
? s s1 and s4
             53  S1
        1718060  S4
      S5      18  S1 AND S4
? rd

>>>Duplicate detection is not supported for File 340.

>>>Records from unsupported files will be retained in the RD set.
      S6       9  RD  (unique items)
? t s6/3,k,ab/1-9

 6/3,K,AB/1     (Item 1 from file: 155)
```

12574060 PMID: 10521801

HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells.

Beviglia L; Kramer R H

Department of Stomatology, University of California, San Francisco, San Francisco, CA, USA.

International journal of cancer. Journal international du cancer (UNITED STATES) Nov 26 1999, 83 (5) p640-9, ISSN 0020-7136--Print
Journal Code: 0042124

Contract/Grant No.: DE 11436; DE; NIDCR

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

Expression of hepatocyte growth factor (HGF) and its tyrosine kinase receptor, c-Met , is positively correlated with breast carcinoma progression. We found that in invasive and metastatic MTLn3 breast carcinoma cells, HGF stimulated both initial adhesion to and motility on the extracellular matrix (ECM) ligands laminin 1, type I collagen, and fibronectin. Next, analysis with function-perturbing antibodies showed that adhesion to the different ECM proteins was mediated through specific beta1 integrins. In MTLn3 cells, HGF induced rapid tyrosine phosphorylation and activation of both c-Met and focal adhesion kinase (FAK). Cell anchorage and adhesion to the ECM substrates was required for HGF-induced FAK activation, since HGF failed to trigger tyrosine phosphorylation of FAK in suspended cells. Our results provide evidence that the 2 signaling pathways, integrin/ECM and c-Met/HGF, cooperate synergistically to induce FAK activation in an adhesion-dependent manner, leading to enhanced cell adhesion and motility. Moreover, we found that a FRNK (the FAK-related non-kinase)-like molecule is expressed in MTLn3 cells. Since FRNK acts as a competitive inhibitor of FAK function, our results suggest that a FRNK-like protein could facilitate disassembly of focal adhesions and likely be responsible for the HGF-induced scattering and motility of MTLn3 cells. Copyright 1999 Wiley-Liss, Inc.

... ***1999*** ,

Expression of hepatocyte growth factor (HGF) and its tyrosine kinase receptor, c-Met , is positively correlated with breast carcinoma progression. We found that in invasive and metastatic MTLn3 breast carcinoma cells, HGF stimulated both initial adhesion to and motility on the extracellular matrix (ECM) ligands laminin 1, type I collagen, and fibronectin. Next, analysis with function-perturbing antibodies showed that adhesion to the different ECM proteins was mediated through specific beta1 integrins. In MTLn3 cells, HGF induced rapid tyrosine phosphorylation and activation of both c-Met and focal adhesion kinase (FAK). Cell anchorage and adhesion to the ECM substrates was required...

... suspended cells. Our results provide evidence that the 2 signaling pathways, integrin/ECM and c-Met/HGF, cooperate synergistically to induce FAK activation in an adhesion-dependent manner, leading to enhanced ...

; Animals; Antibodies--pharmacology--PD; Cell Adhesion; Cell Adhesion Molecules--metabolism--ME; Cell Movement; Dose-Response Relationship, Drug; Enzyme Activation--drug effects--DE; Epithelial Cells --drug effects--DE; Extracellular Matrix Proteins; Focal Adhesion Kinase 1; Focal Adhesion Protein-Tyrosine Kinases; Integrins--antagonists and inhibitors...

Chemical Name: Antibodies; Cell Adhesion Molecules; Extracellular Matrix Proteins; Integrins; Laminin; Neoplasm Proteins; laminin 1; Tyrosine; Hepatocyte Growth Factor; Focal Adhesion Kinase...

9/3,K,AB/5 (Item 5 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

12570244 PMID: 10520577

Presence and tyrosine phosphorylation of c-met receptor in human sperm.

Herness E A; Naz R K

Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo 43614-5806, USA.

Journal of andrology (UNITED STATES) Sep-Oct 1999, 20 (5) p640-7, ISSN 0196-3635--Print Journal Code: 8106453

Contract/Grant No.: HD24425; HD; NICHD

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

The c-met receptor is a p190MET tyrosine kinase proto-oncoprotein that through its binding to its ligand, designated hepatocyte growth factor (HGF), induces mitogenic, motogenic, and morphogenic activities in a variety of cell types. The present study was conducted to examine whether or not the c-met receptor is expressed and tyrosine phosphorylated in the human sperm cell. The Western blot analysis, using a monoclonal antibody (MAb2) directed against the extracellular domain of the c-met receptor, showed a specific band of 195 kDa corresponding to the intact c-met receptor in the detergent-solubilized human sperm preparation (HSP). This protein band was not recognized by the control myeloma lg (immunoglobin). In the immunoprecipitation procedure, a similar specific band of 195 kDa and a 145-kDa band corresponding to the beta-subunit of c-met receptor were seen. In the indirect immunofluorescence technique, the c- ***met*** receptor was localized predominantly in the acrosomal region of the sperm cell. The c- ***met*** receptor was tyrosine phosphorylated/autophosphorylated during capacitation and in the cell-free in vitro kinase assay. Incubation of human sperm with hepatocyte growth factor (HGF) or MAb2 to c-met receptor enhanced the degree of tyrosine phosphorylation/autophosphorylation of the c-met receptor up to 5.1-fold. These findings indicate that the c- ***met*** receptor is present in the acrosomal region of human sperm cell and is tyrosine phosphorylated, which is enhanced by HGF and the receptor ***antibody*** . The c- ***met*** system may have an important role in sperm function.

Presence and tyrosine phosphorylation of c-met receptor in human sperm.

... ***1999*** ,

The c-met receptor is a p190MET tyrosine kinase proto-oncoprotein that through its binding to its ligand, designated hepatocyte growth factor (HGF), induces...

...of cell types. The present study was conducted to examine whether or not the c-met receptor is expressed and tyrosine phosphorylated in the human sperm cell. The Western blot analysis, using a monoclonal antibody (MAb2) directed against the extracellular domain of the c-met receptor, showed a specific band of 195 kDa corresponding to the intact c-met receptor in the detergent-solubilized human sperm preparation (HSP). This protein band was not recognized...

...of 195 kDa and a 145-kDa band corresponding to the beta-subunit of c- ***met*** receptor were seen. In the indirect immunofluorescence technique, the c-met receptor was localized predominantly in the acrosomal

region of the sperm cell. The c- ***met*** receptor was tyrosine phosphorylated/autophosphorylated during capacitation and in the cell-free in vitro kinase assay. Incubation of human sperm with hepatocyte growth factor (HGF) or MAb2 to c-met receptor enhanced the degree of tyrosine phosphorylation/autophosphorylation of the c-met receptor up to 5.1-fold. These findings indicate that the c- ***met*** receptor is present in the acrosomal region of human sperm cell and is tyrosine phosphorylated, which is enhanced by HGF and the receptor ***antibody*** . The c- ***met*** system may have an important role in sperm function.

Descriptors: *Proto-Oncogene Proteins c-met--metabolism--ME; *Spermatozoa--metabolism--ME; *Tyrosine--metabolism--ME

Enzyme No.: EC 2.7.1.112 (Proto-Oncogene Proteins c- ***met***)

Chemical Name: Tyrosine; Proto-Oncogene Proteins c-met

?

```
? ds

Set      Items   Description
S1      161999   MET
S2     1718060   ANTIBOD?
S3        6856   S1 AND S2
S4      525058   EXTRACELLULAR
S5         427   S3 AND S4
S6         288   RD  (unique items)
S7         184   S6 AND PY<=2001
S8      123467   (RECEPTOR(5N)KINASE) OR PROTOONCOGENE
S9          53   S7 AND S8
? s hgf
     S10   14687  HGF
? s s10 and s9
           14687  S10
              53  S9
     S11      32  S10 AND S9
? t s11/3,k,ab/20-32
```

02103874 Genuine Article#: KA856 Number of References: 54
Title: OVEREXPRESSION OF THE C-MET HGF RECEPTOR GENE IN HUMAN THYROID CARCINOMAS (Abstract Available)
Author(s): DIRENZO MF; OLIVERO M; FERRO S; PRAT M; BONGARZONE I; PILOTTI S; BELFIORE A; COSTANTINO A; VIGNERI R; PIEROTTI MA; COMOGLIO PM
Corporate Source: UNIV TURIN,SCH MED,DEPT BIOMED SCI & ONCOL/I-10124 TURIN//ITALY/; NATL CANC INST,DEPT EXPTL ONCOL A/MILAN//ITALY/; NATL CANC INST,DEPT PATHOL/MILAN//ITALY/; UNIV CATANIA,SCH MED,DEPT ENDOCRINOL/I-95124 CATANIA//ITALY/
Journal: ONCOGENE, 1992, V7, N12 (DEC), P2549-2553
ISSN: 0950-9232
Language: ENGLISH Document Type: NOTE
Abstract: The receptor for Hepatocyte Growth Factor is a transmembrane tyrosine kinase encoded by the c- ***MET*** oncogene. We have previously shown that the Met protein is expressed in several human epithelial tissues. The receptor is barely ***detectable*** , however, in normal thyroids and in specimens from patients affected by nonneoplastic thyroid diseases. Now we report that the expression of the Met/HGF receptor is increased a hundred fold in 22 out of 41 human carcinomas derived from the thyroid follicular epithelium. A comprehensive analysis of 15 cases showed that the overexpressing carcinomas belong to histotype variants correlated with negative prognosis and in all but one case there were evidences of locally advanced disease and/or distant metastases. The 11 benign adenomas and the 5 medullary carcinomas tested were negative. Western blot analysis with monoclonal antibodies directed against either the intracellular or the extracellular receptor domains failed to reveal major structural alterations. Southern blot analysis also demonstrated that the c-MET gene was not amplified nor rearranged. These data suggest a role for the overexpression of c-MET oncogene in the pathogenesis and progression of thyroid tumors derived from the follicular epithelium.

Detection of MET oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell carcinomas.

Galeazzi E; Olivero M; Gervasio F C; De Stefani A; Valente G; Comoglio P M; Di Renzo M F; Cortesina G

Department of Clinical Physiopathology, University of Turin School of Medicine, Italy.

European archives of oto-rhino-laryngology - official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) - affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery (GERMANY) 1997, 254 Suppl 1 pS138-43, ISSN 0937-4477--Print Journal Code: 9002937

Publishing Model Print

Document type: Journal Article

Languages: ENGLISH

Main Citation Owner: NLM

Record type: MEDLINE; Completed

The c-MET oncogene encodes the receptor for hepatocyte growth factor/scatter factor (HGF /SF), which is known to stimulate the invasive growth of epithelial cells cultured in vitro. The ***Met*** / HGF receptor is a heterodimeric transmembrane tyrosine kinase , which is a prototype for a new family of growth factor receptors. The c- ***MET*** oncogene is expressed in several types of epithelial tissue including keratinocytes and is over-expressed in a number of human carcinomas. Studies on various carcinoma cell lines have shown that over-expression and structural alteration of the receptor result in its activation and confer tumorigenesis. We have studied ***Met*** / HGF receptor expression in tissue specimens from 34 patients with head and neck squamous cell carcinomas (HNSCC) and in 17 regional lymph node metastases. Western blot analysis was employed, using monoclonal antibodies directed against either the intracellular or ***extracellular*** domain of the receptor. Each sample was compared to its normal counterpart. The receptor did not show any major structural alterations in HNSCC tissues, but its expression was increased from 2- to 50-fold in about 70% of tumors. Immunohistochemistry then showed that the same antibodies stained only a few cells in the basal layer of normal squamous epithelium but intensely marked tumor cells. In the lymph node metastases of Met-positive tumors, receptor expression was maintained and sometimes increased with respect to primary tumors. Immunohistochemical analysis of the metastatic lymph nodes showed that cells were negative in the normal lymphatic tissue and strongly stained in tumor cells. Over-expression of the Met/HGF receptor was found at all tumor stages but was more significant in those associated with enlarged or multiple (N2-N3) lymph node metastases. These data show that expression of the Met/HGF receptor may be involved in the progression of HNSCC towards a metastatic phenotype and may be a useful marker of head and neck tumor cell spread to regional lymph nodes.

Detection of MET oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell...

... ***1997*** ,

The c-MET oncogene encodes the receptor for hepatocyte growth factor/scatter factor (HGF /SF), which is known to stimulate the invasive growth of epithelial cells cultured in vitro. The ***Met*** / HGF receptor is a heterodimeric transmembrane tyrosine kinase , which is a prototype for a new family of growth factor receptors. The c- ***MET*** oncogene is expressed in several types of epithelial tissue including keratinocytes and is over-expressed...

... structural alteration of the receptor result in its activation and confer tumorigenesis. We have studied ***Met*** / ***HGF*** receptor expression in tissue specimens from 34 patients with head and neck squamous cell carcinomas (HNSCC) and in 17 regional lymph node metastases. Western blot analysis was employed, using monoclonal antibodies directed

against either the intracellular or extracellular domain of the receptor. Each sample was compared to its normal counterpart. The receptor did...

... 2- to 50-fold in about 70% of tumors. Immunohistochemistry then showed that the same antibodies stained only a few cells in the basal layer of normal squamous epithelium but intensely marked tumor cells. In the lymph node metastases of Met-positive tumors, receptor expression was maintained and sometimes increased with respect to primary tumors. Immunohistochemical...

... in the normal lymphatic tissue and strongly stained in tumor cells.

```
? ds

Set     Items   Description
S1     161999   MET
S2    1718060   ANTIBOD?
S3       6856   S1 AND S2
S4     525058   EXTRACELLULAR
S5        427   S3 AND S4
S6        288   RD  (unique items)
S7        184   S6 AND PY<=2001
S8     123467   (RECEPTOR(5N)KINASE) OR PROTOONCOGENE
S9         53   S7 AND S8
S10     14687   HGF
S11        32   S10 AND S9
? s detect? or diagnos
<------User Break------>
u!
? s detect? or diagnos?
Processing
Processing
         3619441  DETECT?
         4002496  DIAGNOS?
     S12 7071895  DETECT? OR DIAGNOS?
? s s11 and s12
              32  S11
         7071895  S12
     S13       8  S11 AND S12
? t s13/3,k,ab/1-8

 13/3,K,AB/1     (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2007 Dialog. All rts. reserv.

11268602   PMID: 9065649
```

Magnetic resonance imaging studies on nude mice grafted with colorectal adenocarcinoma using ***gadolinium*** -labeled monoclonal antibody.

Saccavini J C; Curtet C; Bohy J; Tellier C; Bourgoin C; Lhoste J M
Oris Industrie, Gif sur Yvette, France.

Investigative radiology (UNITED STATES) Sep 1988, 23 Suppl 1 pS292-3, ISSN 0020-9996--Print Journal Code: 0045377
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

A monoclonal antibody (Ab) 19.9 specific for colorectal carcinoma was labeled with a high number of gadolinium (Gd) atoms for its potential application as a contrast agent in magnetic resonance imaging (***MRI***). The DTPA was ***conjugated*** to 19.9 Ab via the bicyclic DTPA anhydride method (c. DTPA) using c. DTPA/Ab molar ratios between 5 and 150. The aggregates present in great amount at high c. DTPA/Ab ratios were systematically removed. Then the exact number of DTPA effectively conjugated , the immunoreactivity of the resulting 111In-DTPA-Ab were measured. The number of ***DTPA*** ***conjugated*** per ***antibody*** can
be increased 20 to 25 with only a little loss of immunoreactivity. The 19.9 antibody conjugated with 16 and 25 DTPA was labeled with
153GdCl3 for pharmacokinetic studies on xenografted nude mice and with nonradioactive gadolinium to measure ex vivo the effect on the relaxation time T1 of the tumor. We found a 15 to 20% decrease of T1 on the tumor. In vivo experiments using a Bruker system and the same animal model showed a difference in the tumor contrast after the injection of 2 mg of Gd-labeled Ab.

Magnetic resonance imaging studies on nude mice grafted with colorectal adenocarcinoma using ***gadolinium*** -labeled monoclonal antibody.
... ***1988*** ,

```
     ---  -----  -----------
? s gadolinium
      S1   40111  GADOLINIUM
? s mri
      S2  196738  MRI
? s s1 and s2
           40111  S1
          196738  S2
      S3   12205  S1 AND S2
? s (dtpa or diethylenetriaminepentaacetic) (5n)antibod?
           30025  DTPA
            4507  DIETHYLENETRIAMINEPENTAACETIC
         1718356  ANTIBOD?
      S4     763  (DTPA OR DIETHYLENETRIAMINEPENTAACETIC) (5N)ANTIBOD?
? s s3 and s4
           12205  S3
             763  S4
      S5      35  S3 AND S4
? rd

>>>Duplicate detection is not supported for File 340.

>>>Records from unsupported files will be retained in the RD set.
      S6      31  RD  (unique items)
? s s6 and py<=2001
Processing
Processing
              31  S6
        42321723  PY<=2001
      S7      26  S6 AND PY<=2001
? s s7 and py<2001
Processing
              26  S7
        40017829  PY<2001
      S8      23  S7 AND PY<2001
? s bound or attached or conjugated or linked
          570474  BOUND
          722378  ATTACHED
          145510  CONJUGATED
          797766  LINKED
      S9 2144729  BOUND OR ATTACHED OR CONJUGATED OR LINKED
? s s8 and s9
              23  S8
         2144729  S9
     S10      10  S8 AND S9
? t s10/3,k,ab/1-10

 10/3,K,AB/1     (Item 1 from file: 155)
DIALOG(R)File 155:MEDLINE(R)
(c) format only 2007 Dialog. All rts. reserv.

10234155   PMID: 7976589
  MRI contrast enhancement by Gd-DTPA-monoclonal antibody
 in 9L glioma rats.
  Matsumura A; Shibata Y; Nakagawa K; Nose T
  Department of Neurosurgery, University of Tsukuba, Ibaraki, Japan.
  Acta neurochirurgica. Supplementum (AUSTRIA)      ***1994***  ,  60  p356-8,
ISSN 0065-1419--Print   Journal Code: 0140560
  Publishing Model Print
  Document type: Journal Article
  Languages: ENGLISH
  Main Citation Owner: NLM
  Record type: MEDLINE; Completed
```

To achieve a tissue-specific enhancement in diagnosis of brain tumor, a magnetic resonance imaging (MRI) study was performed using conjugate of Gd-DTPA and monoclonal antibody (MoAb) against 9L glioma cells. Fisher 344 strain rats were used for this study. MoAb against 9L glioma cells was conjugated with Gd-DTPA according to the method of Hnatowich et al. (1983) and used for the ***MRI*** study. The gadolinium (Gd) concentration in the Gd-MoAb injected to the rats was 0.01-0.03 mmol/kg. The enhancement effect increased gradually and persisted for 24 hours after the injection. This was longer than Gd-DTPA, which showed a peak of enhancement effect within 30 minutes after injection and was washed out within 120 min. This result was compatible with scintigraphy studies using 125I labeled anti 9L monoclonal antibody, in which the accumulation of the 125I antibody increased at 24, 48 and 72 hours after the injection. By using tumor-specific contrast agents such as Gd-MoAb, it may be possible to differentiate among tumor, perifocal edema and radiation injury.

MRI contrast enhancement by Gd-DTPA-monoclonal antibody in 9L glioma rats.
... ***1994*** ,
To achieve a tissue-specific enhancement in diagnosis of brain tumor, a magnetic resonance imaging (MRI) study was performed using conjugate of Gd-DTPA and monoclonal antibody (MoAb) against 9L glioma cells. Fisher 344 strain rats were used for this study. MoAb against 9L glioma cells was conjugated with Gd-DTPA according to the method of Hnatowich et al. (1983) and used for the ***MRI*** study. The gadolinium (Gd) concentration in the Gd-MoAb injected to the rats was 0.01-0.03...
; Animals; Antibodies, Monoclonal--immunology--IM; Antibody Specificity; Brain--pathology--PA; Brain Neoplasms--immunology--IM; Gadolinium DTPA; Glioma--immunology--IM; Neoplasm Transplantation; Organometallic Compounds--pharmacokinetics--PK; Pentetic Acid--diagnostic use--DU...
Chemical Name: Antibodies, Monoclonal; Contrast Media; Organometallic Compounds; Pentetic Acid; Gadolinium DTPA

10/3,K,AB/2 (Item 2 from file: 155)
DIALOG(R)File 155:MEDLINE(R)

09053873 PMID: 1722486
AUR Memorial Award 1991. Immunogenicity of ***gadolinium*** -based contrast agents for magnetic resonance imaging. Induction and characterization of antibodies in animals.
Baxter A B; Melnikoff S; Stites D P; Brasch R C
Department of Radiology, University of California, San Francisco 94143-0628.
Investigative radiology (UNITED STATES) Dec 1991, 26 (12) p1035-40, ISSN 0020-9996--Print Journal Code: 0045377
Contract/Grant No.: CA 09386; CA; NCI; CA 49786; CA; NCI
Publishing Model Print
Document type: Journal Article
Languages: ENGLISH
Main Citation Owner: NLM
Record type: MEDLINE; Completed

NPL X ADONIS___ MIC___ BioT___Main__NO__Vol NO__ NOS___CKCite__Dup__Int SAS 4-2-07

STIC-ILL

From: Davis, Minh-Tam
Sent: Monday, April 02, 2007 4:06 PM
To: STIC-ILL
Subject: Reprint request for 10/500297

1) Presence and tyrosine phosphorylation of c-met receptor in human sperm.
Herness E A; Naz R K
Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo 43614-5806, USA.
Journal of andrology (UNITED STATES) Sep-Oct 1999, 20 (5) p640-7, ISSN 0196-3635--Print Journal Code: 8106453
Contract/Grant No.: HD24425; HD; NICHD

2) HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells.
Beviglia L; Kramer R H
Department of Stomatology, University of California, San Francisco, San Francisco, CA, USA.
International journal of cancer. Journal international du cancer (UNITED STATES) Nov 26 1999, 83 (5) p640-9, ISSN 0020-7136--Print
Journal Code: 0042124

3) Title: OVEREXPRESSION OF THE C-MET HGF RECEPTOR GENE IN HUMAN THYROID CARCINOMAS (Abstract Available)
Author(s): DIRENZO MF; OLIVERO M; FERRO S; PRAT M; BONGARZONE I; PILOTTI S; BELFIORE A; COSTANTINO A; VIGNERI R; PIEROTTI MA; COMOGLIO PM
Corporate Source: UNIV TURIN,SCH MED,DEPT BIOMED SCI & ONCOL/I-10124 TURIN//ITALY/; NATL CANC INST,DEPT EXPTL ONCOL A/MILAN//ITALY/; NATL CANC INST,DEPT PATHOL/MILAN//ITALY/; UNIV CATANIA,SCH MED,DEPT ENDOCRINOL/I-95124 CATANIA//ITALY/
Journal: ONCOGENE, 1992, V7, N12 (DEC), P2549-2553
ISSN: 0950-9232

4) 268602 PMID: 9065649
Detection of MET oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell carcinomas.
Galeazzi E; Olivero M; Gervasio F C; De Stefani A; Valente G; Comoglio P M; Di Renzo M F; Cortesina G
Department of Clinical Physiopathology, University of Turin School of Medicine, Italy.
European archives of oto-rhino-laryngology - official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) - affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery (GERMANY) 1997, 254 Suppl 1 pS138-43, ISSN 0937-4477--Print Journal Code: 9002937

THANK YOU.

MINH TAM DAVIS
ART UNIT 1642, REMSEN 3A24, MB 3C18
272-0830

Journal of Andrology, Vol. 20, No. 5, September/October 1999

Presence and Tyrosine Phosphorylation of c-met Receptor in Human Sperm

ELIZABETH A. HERNESS AND RAJESH K. NAZ

From the Division of Research, Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo, Ohio.

ABSTRACT: The c-met receptor is a p190^{MET} tyrosine kinase proto-oncoprotein that through its binding to its ligand, designated hepatocyte growth factor (HGF), induces mitogenic, motogenic, and morphogenic activities in a variety of cell types. The present study was conducted to examine whether or not the c-met receptor is expressed and tyrosine phosphorylated in the human sperm cell. The Western blot analysis, using a monoclonal antibody (MAb_2) directed against the extracellular domain of the c-met receptor, showed a specific band of 195 kDa corresponding to the intact c-met receptor in the detergent-solubilized human sperm preparation (HSP). This protein band was not recognized by the control myeloma Ig (immunoglobin). In the immunoprecipitation procedure, a similar specific band of 195 kDa and a 145-kDa band corresponding to the β-subunit of c-met receptor were seen. In the indirect immunofluorescence technique, the c-met receptor was localized predominantly in the acrosomal region of the sperm cell. The c-met receptor was tyrosine phosphorylated/autophosphorylated during capacitation and in the cell-free *in vitro* kinase assay. Incubation of human sperm with hepatocyte growth factor (HGF) or MAb_2 to c-met receptor enhanced the degree of tyrosine phosphorylation/autophosphorylation of the c-met receptor up to 5.1-fold. These findings indicate that the c-met receptor is present in the acrosomal region of human sperm cell and is tyrosine phosphorylated, which is enhanced by HGF and the receptor antibody. The c-met system may have an important role in sperm function.

Key words: Testis, fertilization, scatter factor/hepatocyte growth factor, infertility, cytokines.

J Androl 1999;20:640–647

Protein tyrosine phosphorylation has a definite role in a variety of cellular phenomena, including regulation of function of various receptors (Ullrich and Schlessinger, 1990). The receptors for several growth factors are themselves tyrosine protein kinases that regulate cell proliferation and differentiation (Ullrich and Schlessinger, 1990). All receptor tyrosine kinases possess a large, glycosylated, extracellular ligand-binding domain, a single hydrophobic transmembrane region, and a cytoplasmic domain that contains the tyrosine kinase activity (Ullrich and Schlessinger, 1990; Smith et al, 1993; Pawson, 1995). Tyrosine phosphorylation may be the primary or even the exclusive indication of a signal transduction pathway. Other laboratories and ours have demonstrated a vital role of tyrosine phosphorylation in the development of fertilizing capacity of human sperm (Naz et al, 1991; Naz and Ahmad, 1994; Ahmad and Naz, 1995; Leclerc et al, 1996; Naz, 1996; Luconi et al, 1998).

The c-met proto-oncogene encodes a transmembrane glycoprotein, p190^{MET} tyrosine kinase receptor, which is cleaved posttranslationally into an α chain (50 kD) that is a part of extracellular domain and into the disulfide-linked β chain (145 kD) that consists of an extracellular portion involved in ligand binding, a membrane-spanning segment, and a cytoplasmic tyrosine kinase domain (Park et al, 1987; Giordano et al, 1988). The ligand for the c-met receptor has been identified (Bottaro et al, 1991), and it is designated scatter factor (SF)/hepatocyte growth factor (HGF), a protein with structural similarities to plasminogen (Naldini et al, 1991a).

SF/HGF contains four characteristic kringle domains at the NH_2 terminus and a serine protease–like domain at the COOH terminal (Nakamura et al, 1989). SF/HGF has been purified and identified as a heterodimer of heavy (58 kD) and light (31 kD) disulfide-linked subunits. Human and rat HGFs have been cloned and sequenced, and their amino acid sequences have been deduced from the cDNA sequences (Nakamura et al, 1989; Tashiro et al, 1990). SF/HGF has potent mitogenic, motogenic, and morphogenetic activities on epithelial cells in the *in vitro* assays (Bhargava et al, 1991; Matsumoto and Nakamura, 1991; Bussolino et al, 1992; Cooper, 1992; Strain, 1993). SF/HGF-like proteins have been identified in cultured fibroblasts; arterial smooth-muscle cell–conditioned medium; human amniotic fluid; human placental tissue; and in the stromal components of human testis, prostate, mammary gland, uterine endometrium, and ovary (Giordano et al, 1989; Zarnegar et al, 1990; Bhargava et al, 1991; Di Renzo et al, 1991; Naldini et al, 1991b; Lail-Trecker et al,

Supported in part by NIH grant HD24425 to R.K.N.

Correspondence to: Rajesh K. Naz, Ph.D., Professor and Director, Division of Research, Health Education Building, Room 211, Medical College of Ohio, 3055 Arlington Ave., Toledo, Ohio 43614–5806. E-mail: rnaz@mco.edu

Received for publication February 26, 1999; accepted for publication April 20, 1999.

1998). The c-met receptor has been immunochemically localized in the human seminiferous epithelium and sperm cells (Depuydt et al, 1996). Our laboratory demonstrated that the SF/HGF is differentially expressed in various segments of the male genital tract, with the highest expression in cauda and distal corpus parts of epididymis in the mouse (Naz et al, 1994). This region-specific expression coincides with the pattern of acquiring the fertilizing capacity of sperm cell during its transit through the male genital tract.

In view of the above findings, the present study was conducted to examine 1) if the human sperm cell has expression of the c-met receptor, 2) if yes, what its molecular identity is, and 3) whether or not it is tyrosine phosphorylated, since the tyrosine phosphorylation has been shown to have a vital role in the development of fertilizing capacity of sperm cells.

Materials And Methods

Sperm Collection

Human sperm were collected from healthy, fertile men (n = 7) after 48–72 hours of sexual abstinence. Ejaculated semen was liquefied for 1 hour at 37°C and analyzed for volume, sperm concentration, percent motility, and progressive motility (Naz, 1987; Naz et al, 1989). Semen samples with a concentration of >50 × 10^6 sperm/ml and with motility >60%, progressive motility >3 (on a scale of 0–5), and contamination by immature germ cells and immune cells of <1% of total sperm concentration were used in the present study. Sperm were washed twice with modified Biggers, Whitten, and Whittingham (BWW) medium (21 mM HEPES buffer with 4 mM sodium bicarbonate, supplemented with 1% bovine serum albumin [BSA]; Irvine Scientific, Santa Ana, California), and subjected to the swim-up procedure in the same medium (Naz, 1987). The swim-up sperm population was obtained by overlaying the washed sperm pellet with 1.0 ml of supplemented modified BWW medium and incubating the tube in a 20°-angled rack at 37°C in 5% CO_2 in air for 1 hour. The supernatant containing the swim-up population was harvested by careful aspiration; analyzed for motility, concentration, and any contamination; and used in the following procedures. Only those samples that were without any contamination of immature germ cells and leukocytes were used.

The fertile men used in the present study were donor controls (ages 24–32 years) from our andrology laboratory. Sperm from these men undergo capacitation within 5 hours of incubation in the above medium and conditions (Naz, 1987; Ahmad and Naz, 1995). Approximately 10–18% of sperm undergo spontaneous acrosome reaction after 5 hours of capacitation, and 46–68% undergo induced acrosome reaction after treatment (at 37°C for 1 hour) with 10 μM final concentration of A23187 calcium ionophore (Sigma Chemical Co., St. Louis, Missouri). Also, sperm from these men show excellent penetration of zona-free hamster oocytes (100% of ova penetrated; 15–28 sperm per egg).

Recombinant Human SF/HGF and Antibodies

The human recombinant hepatocyte growth factor/scatter factor (HGF/SF) was obtained from Sigma. It was made by using the *Sf* 21 insect cell expression system, and biological activity was measured by its ability to stimulate ^{3}H-thymidine incorporation in the monkey epithelial cell line 4MBr–5 (Rubin et al, 1991).

Two different monoclonal antibodies (MAbs) were used to detect the c-met receptor present in the human sperm (Upstate Biotechnology, Lake Placid, New York). Both of these antibodies were derived from mouse hybridomas raised against the extracellular domain of the c-met receptor present in the human gastric carcinoma cell line GTL-16. One of these antibodies (IgG_{2a}, isotype; clone DO-24, designated MAb_1) works well in the immunoprecipitation procedure, and the other ($IgG1_\kappa$ isotype; clone DL-21, designated MAb_2) is well suited for the Western blot procedure.

The anti-phosphotyrosine mouse PY-20 monoclonal antibody (IgG_{2b}) used in this experiment was purchased from Signal Transduction Laboratories (Lexington, Kentucky). This antibody reacts specifically with phosphotyrosine residues and does not cross-react with phosphoserine or phosphothreonine residues (Frackelton et al, 1983). The affinity-purified monoclonal antibody of IgG_{2b} subclass obtained from Organon-Teknika Corporation (Durham, North Carolina) was used as control immunoglobulins (Ig).

Detection of c-met Receptor

The presence of c-met receptor in human sperm cell was examined by using the Western blot procedure, immunoprecipitation procedure, and indirect immunofluorescence technique. These are described below.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis/Western Blot Procedure—Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)/Western blot procedure was performed using the swim-up sperm population (Naz et al, 1986; Naz et al, 1993). Briefly, the sperm were washed three times with phosphate-buffered saline (PBS), and the sperm membrane proteins were solubilized (at 4°C, overnight) with 0.5% Nonidet P-40 and 0.02% sodium azide in PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF). The solubilized-sperm preparation (HSP) was processed by centrifuge, and the supernatant (80–150 μg of protein) was boiled for 5 minutes in nonreducing sample buffer (Laemmli, 1970) and resolved in a 5–15% gradient gel (Naz et al, 1986).

After SDS-PAGE, the proteins were electrophoretically (at 100 mA for 18 hours at room temperature) transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, New Hampshire; Towbin et al, 1979). The membranes were blocked with 3% BSA, and the proteins were immunodetected by first incubating the blots with c-met MAb_2/control myeloma Ig and then with alkaline phosphatase–conjugated, goat affinity-purified, anti-mouse antibodies (Cappel/Teknika Corp, West Chester, Pennsylvania). The reacted proteins were visualized with nitroblue tetrazolium (NBT) with 5-bromo-4-chloro-3-indolyl phosphate as a substrate (Naz et al, 1993). Finally, the membranes were washed in distilled water, dried, and photographed.

Immunoprecipitation Procedure—The presence of the c-met receptor was also examined by using the immunoprecipitation

procedure, as described in detail elsewhere (Naz et al, 1996). Briefly, HSP (80–150 μg protein), prepared as described above, was mixed with c-met MAb_1/control myeloma Ig (10 μg/100 μl sperm suspension) and incubated overnight at 4°C. Then 20 μl of protein G Plus/Protein A agarose beads (Oncogene Research Products/Calbiochem, Cambridge, Massachusetts) were added to the reaction mixture and incubated at 4°C in a shaker for an additional 1.5 hours. The reaction mixture was processed by centrifuge (at 8,000 × *g* for 5 minutes), and the supernatant was discarded. The pellet was washed three times with radioimmunoprecipitation assay (RIPA) buffer (50 mM NaCl, 10 mM Tris, 1 mM ethylenediaminetetracetic acid [EDTA], 1 mM PMSF, and 0.1% Triton X-100). After washing, the pellet was boiled for 10 minutes in SDS sample buffer (nonreduced) and processed by centrifuge, and the supernatant was resolved by SDS-PAGE (5–15% gradient), and the gel was stained with silver nitrate (BioRad, Richmond, California).

Indirect Immunofluorescence Technique—For indirect immunofluorescence technique (IFT), swim-up sperm were collected, the concentration was adjusted to 1 × 10^6 sperm/ml, and 20 μl of the sperm suspension were applied into wells of immunofluorescence slides (Naz et al, 1991). The slides were air-dried, fixed in methanol for 30 minutes at room temperature, and air-dried again. The nonspecific-binding sites were blocked by incubating the fixed sperm with 0.3% BSA in PBS for 45 minutes. The slides were washed and then incubated for 1 hour with c-met MAb_2/control myeloma Ig (0.1–0.2 μg/well) at room temperature in a humidified chamber. The slides were washed three times again with PBS and incubated for 1 hour with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (1:20 dilution in PBS). The slides were then washed with PBS again, a drop of mounting medium (90% glycerol in PBS containing 0.1% sodium azide and 10 mg/ml of 1,4-diazabicyclo [2,2,2] octane) was applied into each well, and the slides were covered with a coverslip and examined with a fluorescence microscope.

Tyrosine Phosphorylation of c-met Receptor

To examine whether or not the c-met receptor present in human sperm cell is tyrosine phosphorylated or not, both intact and cell-free *in vitro* systems were used. We describe both in the following sections.

Intact In Vitro System—For the intact *in vitro* system, the swim-up sperm (10–20 ×10^6 sperm/ml) were incubated (at 37°C in 5% CO_2 in air) for 0, 15, and 30 minutes with or without HGF (50 ng/ml) or c-met MAb_2/control myeloma Ig (1:10 dilution) and then membrane solubilized (at 4°C, overnight) in NP-40 lysis buffer, as described above. The solubilized-sperm preparation (80–150 μg of protein) was incubated (at 4°C, overnight) with c-met MAb_1/control myeloma Ig, and then 20 μl of protein G Plus/Protein A agarose beads were added to the reaction mixture and incubated (at 4°C for 1.5 hours). The reaction mixture was processed by centrifuge (at 8,000 × *g* for 5 minutes), the supernatant discarded, and the pellet washed three times with RIPA buffer and boiled for 5 minutes in nonreduced SDS-buffer. The proteins were resolved in SDS-PAGE and then transferred to nitrocellulose sheets for Western blot analysis. The membranes were blocked with 3% BSA, and the tyrosine-phosphorylated proteins were immunodetected by incubating the blots first with anti-phosphotyrosine PY-20 MAb and then with alkaline phosphatase–conjugated, goat affinity-purified, anti-mouse antibodies. The reacted protein bands were visualized with NBT and 5-bromo-4-chloro-3-indolyl phosphate. The intensity of various bands was compared by using the Scion Image analysis system (National Center for Biotechnology Information, National Institutes of Health, Bethesda, Maryland).

Cell-Free In Vitro Kinase Assay—The *in vitro* kinase assay was carried out as described elsewhere (Naz et al, 1991; Naz and Ahmad, 1994). Briefly, the swim-up sperm were collected and the membrane proteins were solubilized (at 4°C, overnight) in NP-40 lysis buffer as described above. The solubilized-sperm preparation was reacted with c-met MAb_1/control myeloma Ig at 4°C overnight and then incubated (at 4°C for 1 hour) with protein G Plus/Protein A agarose beads. The reaction mixture was processed by centrifuge (8,000 × *g* for 5 minutes), the supernatant was discarded, and the pellet was washed three times with RIPA buffer. The *in vitro* kinase reaction was carried out on the immunocomplexed beads at 4°C in a total volume of 40 μl of kinase buffer (20 mM HEPES, pH 7.5; 150 mM NaCl; 0.1% Triton X-100; 10% glycerol; 5 mM manganese chloride; 1 mM magnesium chloride; and 10 μCi γ-^{32}P adenosine triphosphate [ATP]). The reaction was stopped by adding nonreduced SDS sample buffer. Proteins were separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane for Western blot analysis using anti-phosphotyrosine PY-20 MAb. The reacted proteins were visualized, and the blot was subjected to autoradiography. The protein bands detected by PY-20 MAb were compared for molecular identities with the radiolabeled bands detected in the autoradiogram.

Results

Detection of c-met Receptor

In the Western blot procedure, the c-met MAb_2 specifically recognized a single band of 195 kDa on the Western blot of detergent-solubilized human sperm preparation (Fig. 1, Panel A) that was not recognized by the control myeloma Ig (Fig. 1, Panel B). In the immunoprecipitation procedure, the c-met MAb_1 specifically immunoprecipitated the protein bands corresponding to the intact c-met receptor (195 kD) and its β subunit (145 kD) (Fig. 2). In addition, there was another band of 45–50 kDa specifically immunoprecipitated by the MAb_1 (Fig. 2). These bands were not immunoprecipitated by the control myeloma Ig. In IFT, the c-met MAb_2 reacted predominantly with the acrosomal regions of ≈70–80% of methanol-fixed human sperm (Fig. 3b). In ≈10% of sperm, the antibody also reacted with the tail regions. The control myeloma Ig did not react with methanol-fixed sperm (Fig. 3d). Even after increasing the concentration up to 2 μg/well and increasing the time of incubation of antibodies with sperm up to 4–6 hours, the fluorescence was always of weak intensity, as shown in Figure 3.

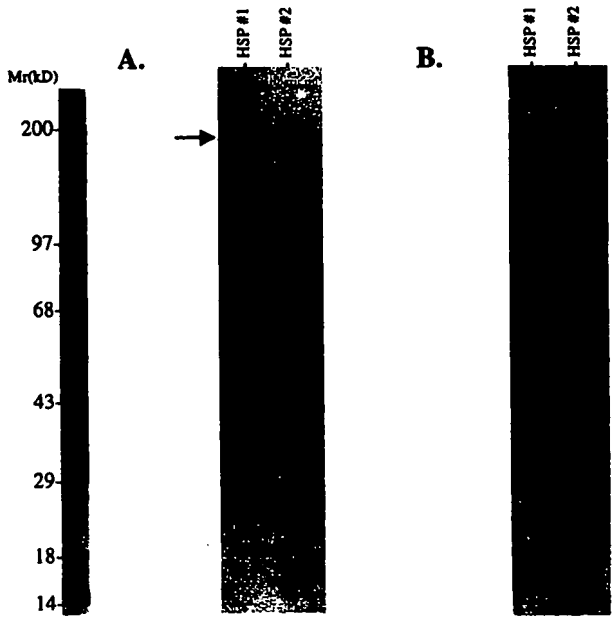

FIG. 1. Western blot analysis of detergent-solubilized human sperm preparation (HSP) using c-met receptor monoclonal antibody MAb_2. The 195-kDa protein band corresponding to intact c-met receptor was recognized in HSP by the c-met MAb_2 (Panel A). The control myeloma Ig did not recognize this band (Panel B). Sperm preparations from seven fertile men were tested, and identical results were obtained. Reaction patterns of HSP from two men are shown here.

Tyrosine Phosphorylation of c-met Receptor

Incubation of sperm cells with HGF (50 ng/ml) increased the tyrosine phosphorylation of c-met receptor (Fig. 4). Both the intact receptor and its β subunit were tyrosine phosphorylated even without the addition of HGF, as is clear at the 0-minute time period that started after the 1-hour swim-up procedure. Addition of HGF increased the tyrosine phosphorylation of the intact receptor by 1.8-fold at 15 minutes of incubation time and by 2.9-fold at 30 minutes of incubation time and increased the tyrosine phosphorylation of the β subunit by 3.1-fold at 15 minutes of incubation time and by 5.1-fold at 30 minutes of incubation time. The tyrosine phosphorylation of at least one other protein band of 45–50 kDa also increased after incubation with HGF.

Surprisingly, incubation of sperm with c-met MAb_2 (1:10 dilution) also increased the tyrosine phosphorylation of intact receptor by 1.7-fold at 15 minutes and by five-fold at 30 minutes of incubation and increased the tyrosine phosphorylation of the β subunit by nil at 15 minutes and by 5.2-fold at 30 minutes of incubation. The tyrosine phosphorylation of at least one other protein band of 45–50 kDa also increased after incubation with c-met MAb_2. The sperm incubated for 30 minutes with BSA or control myeloma Ig did not show an increase in intensity of the receptor proteins compared with that at the 0-minute time point (Fig. 4).

In the cell-free *in vitro* kinase assay carried out on beads using specific MAb_1, the c-met receptor (intact as well as β subunit) was autophosphorylated along with another protein band of 45–50 kDa (Fig. 5, Panel A). These protein bands were also detected by the phosphotyrosine PY-20 MAb, indicating that they were autophosphorylated at the tyrosine residues (Fig. 5, Panel B). There were some additional bands visible on the blots as well as on the autoradiogram; however, these three were the major protein bands detected.

Discussion

Our findings indicate that the c-met receptor is present in the human sperm cell, predominantly in the acrosomal subcellular site. All three methods, namely the Western blot procedure, immunoprecipitation procedure, and indirect immunofluorescence technique, demonstrated its presence. The c-met receptor of 195 kDa observed in the human sperm cell in the Western blot procedure seems to be of the same molecular identity as has been reported in other cell types (Di Renzo et al, 1991; Prat et al, 1991; Humphrey et al, 1995; Yang and Park, 1995; Clark et al, 1996; Kolatsi-Joannou et al, 1997). The immunoreactivity of the MAb_2 with sperm c-met receptor protein was specific since 1) it showed no reaction with any other protein in the Western blot of human sperm preparation, 2) control myeloma Ig of the same class and isotype specificity as MAb_2 did not recognize the 195 kDa band, and 3) sperm preparations from different fertile men showed an identical reaction pattern.

In the immunoprecipiation procedure, the c-met MAb_1 specifically immunoprecipitated the protein bands corresponding to intact (195 kD) and β subunit (145 kD). Since the MAb_1 was raised against the extracellular domain comprising the β subunit of the c-met receptor, the reactivity with both the intact and β subunits was expected. However, the reason for detection of the 145-kDa band, corresponding to the β subunit of the c-met receptor, is not clear. We used nonreducing conditions that should not dissociate the disulfide-linked 145-kDa (β) subunit from the 50-kDa (α) subunit. Similar results were seen with all the sperm preparations from seven fertile men examined, indicating that this was not an experimental error. It is possible that in sperm cells, the c-met receptor is present both as an intact as well as an isolated β subunit. The β subunit is almost a complete unit, as it has an extracellular ligand-binding region, a transmembrane domain, a tyrosine kinase domain, and sites for tyrosine phosphorylation (Bottaro et al, 1991).

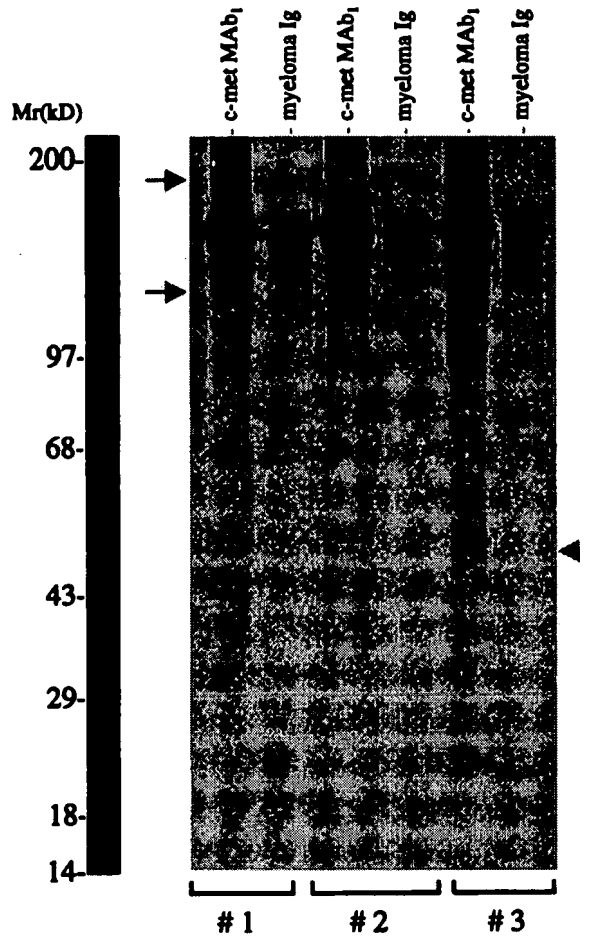

FIG. 2. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis pattern (PAGE) of c-met receptor protein immunoprecipitated from the human sperm preparation. The c-met MAb_1 specifically immunoprecipitated two protein bands, namely 195-kDa and 145-kDa bands corresponding to intact and β subunit of c-met receptor, respectively, and another 45–50 kDa cross-reactive band was also coimmunoprecipitated. These bands were not immunoprecipitated by the control myeloma Ig. Sperm samples from seven fertile men were tested on various days, and an identical pattern was observed. Immunoprecipitation patterns of sperm preparation from three men (#1, 2, and 3) are shown here.

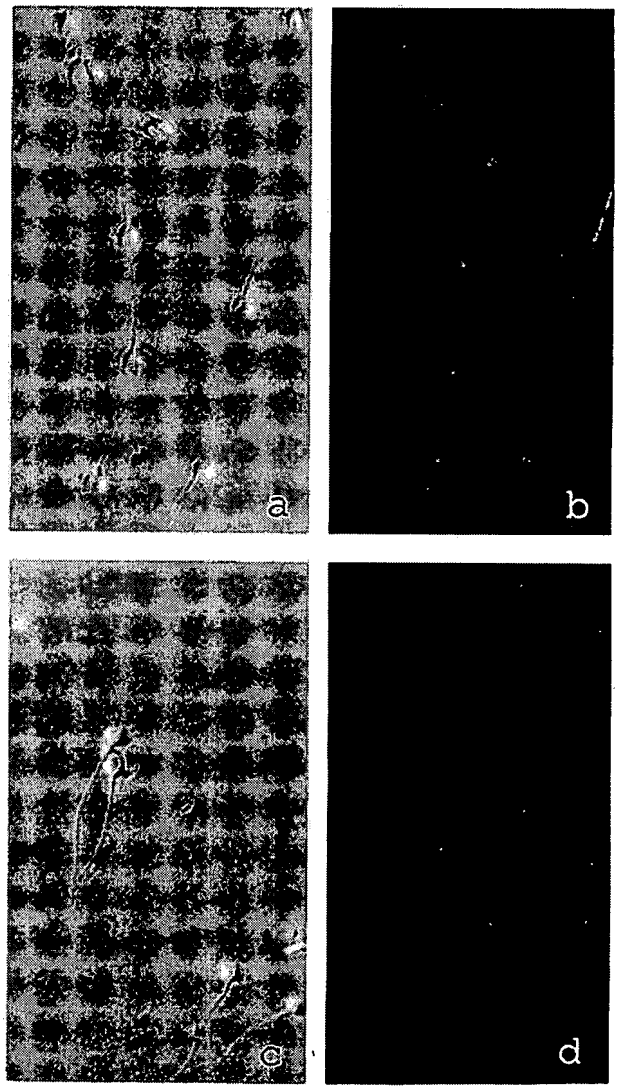

FIG. 3. Immunofluorescent localization of c-met receptor on methanol-fixed human sperm cells. The c-met MAb_1 reacted predominantly with the acrosomal regions of ≈70–80% sperm (b). In ≈10% of sperm, the antibody also reacted with the tail regions. Immunoreactivity of the antibody, although specific, was of weak intensity. Control myeloma Ig did not react with the sperm (d). **a** and **c** are the corresponding phase-contrast pictures of **b** and **d**, respectively. Magnifications, **a–d**: 410×.

In IFT, the c-met receptor was localized in the acrosomal region (majority of the cells) and tail region of the sperm cell. The reactivity of the MAb_2 was of weak intensity, even after using a higher concentration (2 μg/well) and incubating it with sperm for a longer time. The weaker reactivity may be due to a lower density of c-met receptors per sperm cell. Also, the MAb_2 may not be suitable for immunochemical detection of the receptor, since this antibody is primarily used for the Western blot procedure. However, the MAb_1, which is recommended for the immunoprecipitation procedure, also did not increase the intensity of fluorescence in IFT.

The c-met receptor is not only present in sperm cells but is also tyrosine phosphorylated/autophosphorylated during capacitation, and degree of tyrosine phosphorylation/autophosphorylation is enhanced by its ligand HGF/SF as well as by its antibody. In the intact *in vitro* system, HGF enhanced the tyrosine phosphorylation of the receptor protein. Surprisingly, the c-met antibody MAb_2 also enhanced the tyrosine phosphorylation of the receptor and acted as a ligand. Similar increase in intensity of tyrosine phosphorylation of the receptor by its antibodies has been seen in other systems (Kumar et al, 1991; Naz and Ahmad, 1992). The effect of the HGF/receptor antibody on the tyrosine phosphorylation/autophosphorylation was

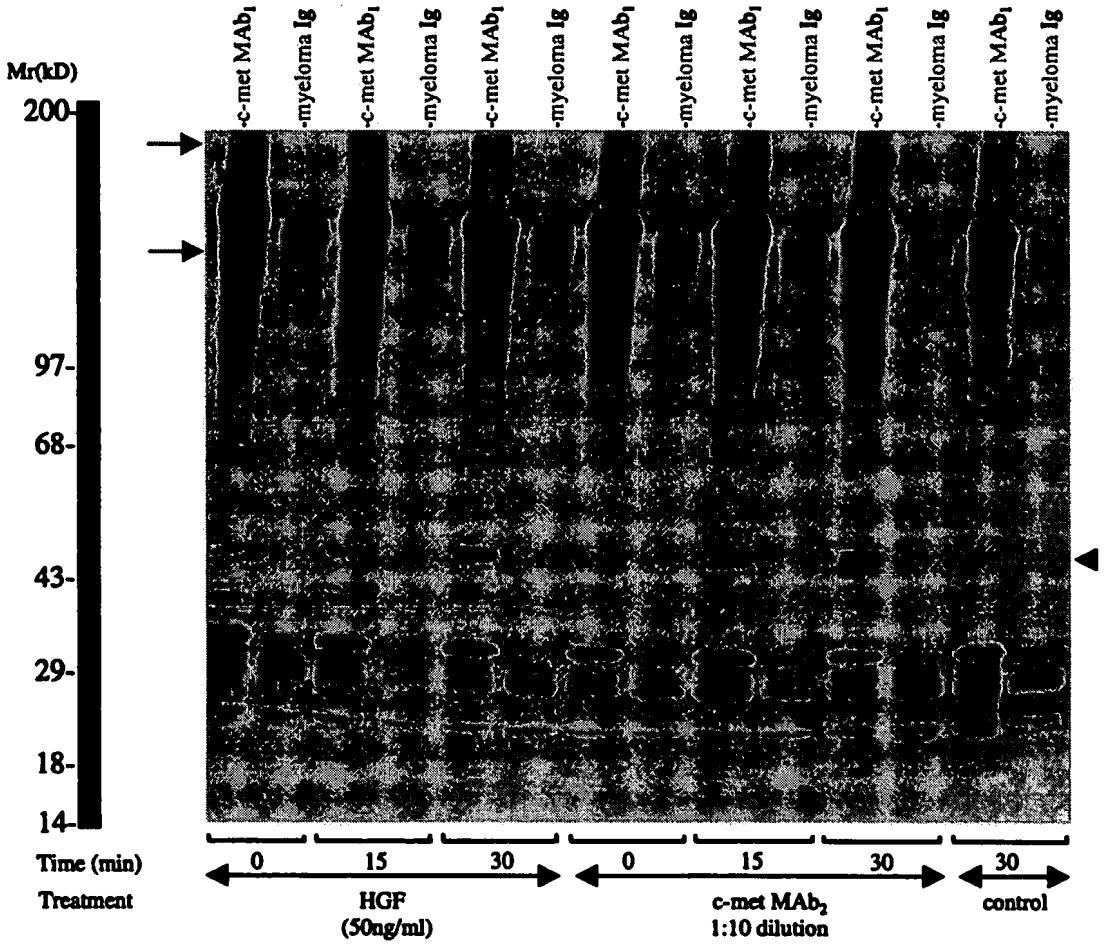

FIG. 4. Western blot analysis indicating the tyrosine phosphorylation pattern of c-met receptor proteins in sperm cells that were incubated for various time periods with or without HGF or c-met MAb_2. After incubation, the sperm membrane proteins were solubilized in NP-40 lysis buffer and immunoprecipitated with c-met MAb_1/control myeloma Ig. The precipitates were resolved in SDS-PAGE and transferred to nitrocellulose sheets for Western blot analysis using anti-phosphotyrosine PY-20 MAb. The addition of HGF or c-met MAb_2 during incubation increased the tyrosine phosphorylation of both the intact as well as the β subunit of c-met receptor over time (shown by arrow). Also, there was an increase in tyrosine phosphorylation of another protein of 45–50 kDa by HGF and c-met MAb_2.

rapid and was seen as early as 15 minutes after incubation. There was some degree of tyrosine phosphorylation/autophosphorylation of the receptor even without the addition of HGF/receptor antibody, indicating that the receptor protein undergoes phosphorylation/autophosphorylation during capacitation by cross-talk through interaction with other molecules (Naz, 1996).

There was at least one other molecule of 45–50 kDa that was tyrosine phosphosphorylated/autophosphorylated during capacitation and whose phosphosphorylation/autophosphorylation was enhanced by the addition of HGF/receptor antibodies. Although it falls in the same molecular range as the α subunit of c-met receptor, it is a different polypeptide, since the α subunit is part of the extracellular domain of c-met receptor and does not have tyrosine kinase activity or the tyrosine phosphorylation sites (Park et al, 1987; Giordano et al, 1988). This molecule coprecipitates with the c-met receptor using the MAb_1 antibody in the immunoprecipitation procedure and is also detected by the anti-phosphotyrosine PY-20 monoclonal antibody. This protein is not recognized by the MAb_2 in the Western blot procedure. In the cell-free *in vitro* kinase assay, both the intact and β subunit of c-met receptor as well as the 45- to 50-kDa protein showed autophosphorylation at the tyrosine residues. The ^{32}P-labeling in the *in vitro* kinase assay indicates *de novo* tyrosine autophosphorylation of these protein molecules.

In conclusion, our findings indicate that the c-met receptor is present in the human sperm cell and undergoes tyrosine phosphorylation. Since the tyrosine phosphorylation of sperm membrane proteins has a vital role in the development of fertilizing capacity, the c-met receptor may be involved in sperm cell function because it is not only present but also undergoes phosphorylation/auto-

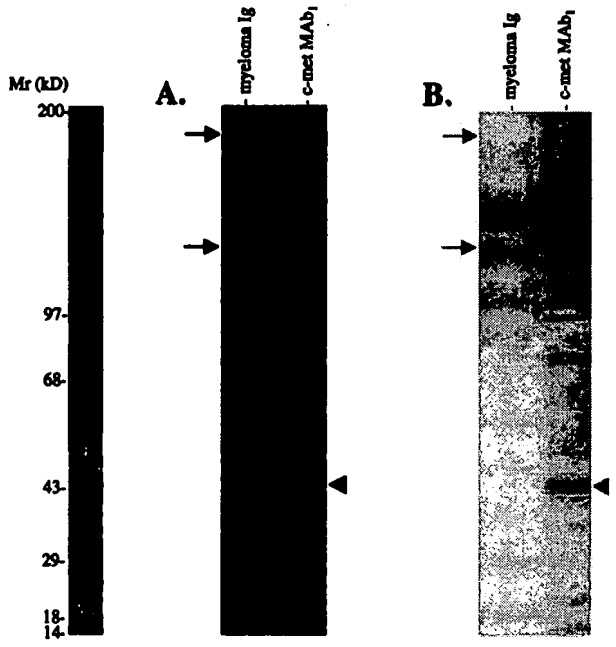

FIG. 5. An autophosphorylation pattern of c-met receptor protein in the *in vitro* kinase assay. The c-met receptor present in the solubilized-sperm preparation was immunoprecipitated by c-met receptor MAb,/control myeloma Ig and the precipitate immunocomplexed onto the protein G Plus/Protein A agarose beads. The *in vitro* kinase assay was carried out on the beads as described in Materials and Methods. The proteins were resolved in SDS-PAGE, transferred to nitrocellulose, autoradiographed **(Panel A)**, and then subjected to Western blot analysis using phosphotyrosine PY-20 MAb **(Panel B)**. Both the intact (195 kD) receptor and its β subunit (145 kD) were autophosphorylated, along with another protein of 45–50 kDa (shown by arrows), as seen by the ^{32}P-labeling of these protein molecules in the autoradiogram **(Panel A)**. All these three molecules were specifically recognized by the PY-20 MAb **(Panel B)**. Although there were additional bands, these three were the major bands detected.

phosphorylation at tyrosine residues. Its interaction with other molecules and the exact signal transduction pathway of its involvement in sperm cell function needs to be elucidated. The present findings on mature sperm cells, along with the previous reports on sperm formation in the testis (Depuydt et al, 1996) and maturation in the epididymis (Naz et al, 1994), indicate that the c-met receptor and its ligand, HGF/SF, may have a role in male fertility. Besides its role in capacitation/acrosome reaction, it may also have an important function in sperm motility since its ligand (SF/HGF) has both mitogenic as well as motogenic properties.

References

Ahmad K, Naz RK. Thymosin alpha-1 and FA-1 monoclonal antibody affect fertilizing capacity of human sperm by modulating protein phosphorylation pattern. *J Reprod Immunol* 1995;29:1–17.

Bhargava MM, Li Y, Joseph A, Pendergast M, Hofmann R, Rosen EM, Goldber ID. Purification, characterization, and mechanism of action of scatter factor from human placenta. In: Goldberg ID, ed. *Cell Motility Factors*. Basel, Switzerland: Birkhauser Verlag; 1991:63–75.

Bottaro DP, Rubin JS, Faletto DL, Chan AL, Kmiecik TE, Vande Woude GF, Aaronson SA. Identification of the hepatocyte growth factor receptor as the c-met proto-oncogene product. *Science* 1991;251:802–804.

Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM. Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. *J Cell Biol* 1992;119:629–641.

Clark DE, Smith SK, Sharkey AM, Sowter HM, Charnock-Jones DS. Hepatocyte growth factor/scatter factor and its receptor c-met: localization and expression in the human placenta throughout pregnancy. *J Endocrinol* 1996;151:459–467.

Cooper CS. The *met* oncogene: from detection by transfection to membrane receptor for hepatocyte growth factor. *Oncogene* 1992;7:3–7.

Depuydt CE, Zalata A, de Potter CR, van Emmelo J, Comhaire FH. The receptor encoded by the human c-met oncogene is expressed in testicular tissue and on human spermatozoa. *Mol Hum Reprod* 1996;2:2–8.

Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM. Expression of the met/HGF receptor in normal and neoplastic human tissues. *Oncogene* 1991;6:1997–2003.

Frackelton AR Jr, Ross AH, Eisen HN. Characterization and use of monoclonal antibodies for isolation of phosphotyrosyl proteins from retrovirus transformed cells and growth factor-stimulated cells. *Mol Cell Biol* 1983;3:1343–1352.

Giordano S, Di Renzo MF, Ferracina R, Chiado-Piat L, Comoglio PM. p145, a protein with associated tyrosine kinase activity in a human gastric carcinoma cell line. *Mol Cell Biol* 1988;8:3510–3517.

Giordano S, Di Renzo MF, Narsimhan RP, Cooper CS, Rosa C, Comoglio PM. Biosynthesis of the protein encoded by the c-met proto-oncogene. *Oncogene* 1989;4:1383–1388.

Humphrey PA, Zhu X, Zarnegar R, Swanson PE, Ratliff TL, Vollmer RT, Day ML. Hepatocyte growth factor and its receptor (c-met) in prostatic carcinoma. *Am J Pathol* 1995;147:386–396.

Kolatsi-Joannou M, Moore R, Winyard PJ, Woolf AS. Expression of hepatocyte growth factor/scatter factor and its receptor, MET, suggests roles in human embryonic organogenesis. *Pediatr Res* 1997;41:657–665.

Kumar R, Shepard HM, Mendelsohn J. Regulation of phosphorylation of the c-erbB-2/HER2 gene product by a monoclonal antibody and serum growth factor(s) in human mammary carcinoma cells. *Mol Cell Biol* 1991;11:979–986.

Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680–685.

Lail-Trecker M, Gulati R, Peluso JJ. A role for hepatocyte growth factor/scatter factor in regulating normal and neoplastic cell of reproductive tissues. *J Soc Gynecol Invest* 1998;5:114–121.

Leclerc P, De Lamirande E, Gagnon C. Cyclic adenosine 3', 5' monophosphate-dependent regulation of protein tyrosine phosphorylation in relation to human sperm capacitation and motility. *Biol Reprod* 1996;55:684–692.

Luconi M, Barni T, Vannelli GB, Krausz C, Marra F, Benedetti PA, Evangelista V, Francavilla S, Properzi G, Forti G, Baldi E. Extracellular signal-regulated kinases modulate capacitation of human spermatozoa. *Biol Reprod* 1998;58:1476–1489.

Matsumoto K, Nakamura T. Hepatocyte growth factor: molecular structure and implications for a central role in liver generation. *Gastroenterol Hepatol* 1991;6:509–519.

Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 1989;342:440–443.

Naldini L, Vigna E, Narsimhan RP, Gaudino G, Zarnegar R, Michalopoulos G, Comoglio PM. Hepatocyte growth factor (HGF) stimulates the tyrosine activity of the receptor encoded by the proto-oncogene c-met. *Oncogene* 1991a;6:501–504.

Naldini L, Weidner M, Vigna E, Gaudino G, Bardelli A, Ponzetto C, Narsimhan RP, Hartmann G, Zarnegar R, Michalopoulos G, Birchmeier W, Comoglio PM. Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J* 1991b; 10:2867–2878.

Naz RK. Involvement of fertilization antigen (FA-1) in involuntary immunoinfertility in humans. *J Clin Invest* 1987;80:1375–1383.

Naz RK. Protein tyrosine phosphorylation and signal transduction during capacitation/acrosome reaction and zona pellucida binding in human sperm. *Arch Androl* 1996;37:47–55.

Naz RK, Ahmad K. Presence of expression products of c-erbB-1 and c-erbB-2/HER2 genes on mammalian sperm cell, and effects of their regulation on fertilization. *J Reprod Immunol* 1992;21:223–239.

Naz RK, Ahmad K. Molecular identities of human sperm proteins that bind human zona pellucida: nature of sperm-zona interaction, tyrosine kinase activity and involvement of FA-1. *Mol Reprod Dev* 1994;39: 397–408.

Naz RK, Ahmad K, Kumar R. Role of membrane phosphotyrosine proteins in human spermatozoal function. *J Cell Sci* 1991;99:157–165.

Naz RK, Ahmad K, Menge AC. Antiidiotypic antibodies to sperm in sera of fertile women that neutralize antisperm antibodies. *J Clin Invest* 1993;92:2331–2338.

Naz RK, Deutsch J, Phillips TM, Menge AC, Fisch H. Sperm antibodies in vasectomized men and their effects on fertilization. *Biol Reprod* 1989;40:163–173.

Naz RK, Joseph A, Lee Y, Ahmad K, Bhargava MM. Expression of scatter factor/hepatocyte growth factor is regionally correlated with the initiation of sperm motility in murine male genital tract: is scatter factor/HGF involved in initiation of sperm motility? *Mol Reprod Dev* 1994;38:431–439.

Naz RK, Morte C, Ahmad K, Martinez P. Hexokinase present in human sperm is not tyrosine phosphorylated but its antibodies affect fertilizing capacity. *J Androl* 1996;17:143–150.

Naz RK, Phillips TM, Rosenblum BB. Characterization of the fertilization antigen-1 for the development of a contraceptive vaccine. *Proc Natl Acad Sci USA* 1986;83:5713–5717.

Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G. Sequence of MET proto-oncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. *Proc Natl Acad Sci USA* 1987;84:6379–6383.

Pawson T. Protein modules and signalling network. *Nature* 1995;373: 573–580.

Prat M, Narsimhan RP, Crepaldi T, Nicotra MR, Natali PG, Comoglio PM. The receptor encoded by the human c-met oncogene is expressed in hepatocytes, epithelial cells and solid tumors. *Int J Cancer* 1991; 49:323–328.

Rubin JS, Chan AM, Bottaro DP, Burgess WH, Taylor WG, Cech AC, Hirschfield DW, Wong J, Miki T, Finch PW. A broad-spectrum human lung fibroblast-derived mitogen is a variant of hepatocyte growth factor. *Proc Natl Acad Sci USA* 1991;88:415–419.

Smith JA, Francis SH, Corbin JD. Autophosphorylation: a salient feature of protein kinases. *Mol Cell Biol* 1993;127/128:51–70.

Strain AJ. Hepatocyte growth factor: another ubiquitous cytokine. *J Endocrinol* 1993;137:1–5.

Tashiro K, Hagiya M, Nishizawa T, Seki T, Shimonishi M, Shimizu S, Nakamura T. Deduced primary structure of rat hepatocyte growth factor and expression of the mRNA in rat tissues. *Proc Natl Acad Sci USA* 1990;87:3200–3204.

Towbin H, Stachelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets, procedures and some applications. *Proc Natl Acad Sci USA* 1979;76:4350–4354.

Ullrich A, Schlessinger J. Signal transduction by receptors with tyrosine kinase activity. *Cell* 1990;61:203–212.

Yang XM, Park M. Expression of the hepatocyte growth factor/scatter factor receptor tyrosine kinase is localized to epithelia of the adult mouse. *Lab Invest* 1995;73:483–491.

Zarnegar R, Muga S, Rahija R, Michalopoulos G. Tissue distribution of hepatopoientin-A: a heparin-binding polypeptide growth factor for hepatocytes. *Proc Natl Acad Sci USA* 1990;87:1252–1256.

NPL X ADONIS___ MIC___ BioT___Main__NO__Vol NO__ NOS___CKCite__Dup__Int SAS 4-2-07

STIC-ILL

From: Davis, Minh-Tam
Sent: Monday, April 02, 2007 4:06 PM
To: STIC-ILL
Subject: Reprint request for 10/500297

1) Presence and tyrosine phosphorylation of c-met receptor in human sperm.
Herness E A; Naz R K
Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo 43614-5806, USA.
Journal of andrology (UNITED STATES) Sep-Oct 1999, 20 (5) p640-7, ISSN 0196-3635--Print Journal Code: 8106453
Contract/Grant No.: HD24425; HD; NICHD

2) HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells.
Beviglia L; Kramer R H
Department of Stomatology, University of California, San Francisco, San Francisco, CA, USA.
International journal of cancer. Journal international du cancer (UNITED STATES) Nov 26 1999, 83 (5) p640-9, ISSN 0020-7136--Print Journal Code: 0042124

3) Title: OVEREXPRESSION OF THE C-MET HGF RECEPTOR GENE IN HUMAN THYROID CARCINOMAS (Abstract Available)
Author(s): DIRENZO MF; OLIVERO M; FERRO S; PRAT M; BONGARZONE I; PILOTTI S; BELFIORE A; COSTANTINO A; VIGNERI R; PIEROTTI MA; COMOGLIO PM
Corporate Source: UNIV TURIN,SCH MED,DEPT BIOMED SCI & ONCOL/I-10124 TURIN//ITALY/; NATL CANC INST,DEPT EXPTL ONCOL A/MILAN//ITALY/; NATL CANC INST,DEPT PATHOL/MILAN//ITALY/; UNIV CATANIA,SCH MED,DEPT ENDOCRINOL/I-95124 CATANIA//ITALY/
Journal: ONCOGENE, 1992, V7, N12 (DEC), P2549-2553
ISSN: 0950-9232

4) 268602 PMID: 9065649
Detection of MET oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell carcinomas.
Galeazzi E; Olivero M; Gervasio F C; De Stefani A; Valente G; Comoglio P M; Di Renzo M F; Cortesina G
Department of Clinical Physiopathology, University of Turin School of Medicine, Italy.
European archives of oto-rhino-laryngology - official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) - affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery (GERMANY) 1997, 254 Suppl 1 pS138-43, ISSN 0937-4477--Print Journal Code: 9002937

THANK YOU.

MINH TAM DAVIS
ART UNIT 1642, REMSEN 3A24, MB 3C18
272-0830

Int. J. Cancer: 83, 640–649 (1999)

Publication of the International Union Against Cancer
Publication de l'Union Internationale Contre le Cancer

HGF INDUCES FAK ACTIVATION AND INTEGRIN-MEDIATED ADHESION IN MTLn3 BREAST CARCINOMA CELLS

Lucia BEVIGLIA[1] and Randall H. KRAMER[2]*

[1]*Department of Stomatology, University of California, San Francisco, San Francisco, CA, USA*
[2]*Departments of Stomatology and Anatomy, University of California, San Francisco, San Francisco, CA, USA*

Expression of hepatocyte growth factor (HGF) and its tyrosine kinase receptor, c-Met, is positively correlated with breast carcinoma progression. We found that in invasive and metastatic MTLn3 breast carcinoma cells, HGF stimulated both initial adhesion to and motility on the extracellular matrix (ECM) ligands laminin 1, type I collagen, and fibronectin. Next, analysis with function-perturbing antibodies showed that adhesion to the different ECM proteins was mediated through specific β1 integrins. In MTLn3 cells, HGF induced rapid tyrosine phosphorylation and activation of both c-Met and focal adhesion kinase (FAK). Cell anchorage and adhesion to the ECM substrates was required for HGF-induced FAK activation, since HGF failed to trigger tyrosine phosphorylation of FAK in suspended cells. Our results provide evidence that the 2 signaling pathways, integrin/ECM and c-Met/HGF, cooperate synergistically to induce FAK activation in an adhesion-dependent manner, leading to enhanced cell adhesion and motility. Moreover, we found that a FRNK (the FAK-related non-kinase)-like molecule is expressed in MTLn3 cells. Since FRNK acts as a competitive inhibitor of FAK function, our results suggest that a FRNK-like protein could facilitate disassembly of focal adhesions and likely be responsible for the HGF-induced scattering and motility of MTLn3 cells. *Int. J. Cancer* 83:640-649, 1999.
© *1999 Wiley-Liss, Inc.*

Hepatocyte growth factor (HGF) has been shown to exert multiple biological activities. It has mitogenic, motogenic and morphogenic effects on various normal and tumor cells (Jiang *et al.*, 1999). However, it has also been shown to inhibit proliferation of some tumor cells (Tajima *et al.*, 1991). HGF binds to its receptor, c-Met, the protein product of the proto-oncogene *c-met*. c-Met is a disulfide-linked heterodimer composed of an alpha subunit (50 kDa) and a beta subunit (145 kDa) which contains intrinsic tyrosine kinase activity. Multiple signaling pathways downstream from c-Met activation account for the observation that HGF stimulates or inhibits biological functions, depending on the cellular environment (Jiang *et al.*, 1999).

There is evidence that HGF may play a role in tumor progression. Several studies have shown a positive correlation between HGF/c-Met levels and invasiveness of various cancer types (Jiang *et al.*, 1999). Also, a limited number of reports suggest an important role for HGF and its receptor c-Met in breast cancer progression (Tuck *et al.*, 1996). Importantly, high levels of HGF in the stroma of the tumor have been correlated with poor survival in breast cancer (Yamashita *et al.*, 1994).

HGF activation of the c-Met tyrosine kinase receptor has been shown to induce cell motility (Jiang *et al.*, 1999). We have shown also that c-Met expression correlates positively with the motile and invasive response of poorly differentiated human breast carcinoma cells to HGF (Beviglia *et al.*, 1997). Only one study (Weimar *et al.*, 1997) has examined the effect of HGF on adhesion of cancer cells to extracellular matrix (ECM) ligands. Cell adhesion to ECM is an important event that precedes cell migration and invasion. In the current study, we examined the highly metastatic rat mammary adenocarcinoma MTLn3 cells for both their motile and their adhesive responses to HGF. We report that MTLn3 cells were stimulated by HGF to adhere as well as to migrate on ECM ligands, including laminin 1, type I collagen and fibronectin. Interestingly, the HGF-induced stimulation of cell adhesion occurred rapidly and was correlated with a specific and significant increase in tyrosine phosphorylation of FAK. This suggests that FAK might play an important role in the HGF/c-Met signaling cascade in MTLn3 cells leading to increased cell adhesion on ECM.

Integrins are important transmembrane cell receptors that mediate cell interactions with ECM (Hynes, 1992). We also show that MTLn3 cells express β1 family integrins; the α2β1 and α3β1 integrins mediate most of the cell adhesion and migration on laminin, and the α2β1 integrin is the major receptor for type I collagen. Integrins are also important transducers of intracellular signaling (Hynes, 1992). FAK plays a central role in integrin-initiated signaling pathways (Burridge *et al.*, 1992) and was reported to be activated also by growth factors (Abedi and Zachary, 1997). Tyrosine phosphorylation of FAK occurs during formation of focal adhesions and spreading (Burridge *et al.*, 1992). We therefore also investigated whether the effects of HGF in MTLn3 cells were integrin dependent. We found that HGF did not stimulate FAK in suspended MTLn3 cells, which suggests that HGF activation of FAK requires integrin engagement. Our results indicate a synergistic effect by the c-Met signaling pathway for integrin-mediated adhesion-dependent stimulation of FAK. The mechanism by which FAK is involved in the HGF-induced cell motility remains less clear than its effect on cell adhesion. We observed that activation of FAK declines over time. Moreover, our results show a significant expression of a FRNK-like molecule that might be upregulated by HGF. The FAK-related non-kinase FRNK, corresponding to the COOH-terminal domain of FAK, has been shown to act as a competitive inhibitor of FAK function since it contains the FAT (focal adhesion targeting) domain. As a separate protein, FRNK is not widely distributed among cells; when overexpressed into chicken embryo cells, it inhibits tyrosine phosphorylation of FAK and determines a delayed cell spreading on fibronectin (Richardson and Parsons, 1996). Morover, microinjection of human umbilical vein endothelial cells with the FAT-containing carboxy terminus of FAK, using the GST fusion protein system, inhibited competitively FAK entry into focal adhesions and DNA synthesis, which indicates a negative effect on cell proliferation (Gilmore and Romer, 1996). Therefore, our results suggest that a FRNK-like molecule we detected on MTLn3 cells could have a role in the HGF induction of cell motility, likely following disassembly of cell focal contacts simultaneously with decrease of FAK tyrosine phosphorylation.

Grant sponsor: National Institutes of Health; Grant number: DE 11436; Grant sponsor: American Italian Cancer Foundation; Grant number: 94-102.

Lucia Beviglia is now at Lineberger Cancer Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599, USA.

*Correspondence to: University of California, Department of Stomatology, HSW604, San Francisco, CA 94143-0512 USA. Fax: +415 4764204; E-mail: randyk@itsa.ucsf.edu

Received 2 March 1999; Revised 14 June 1999

MATERIAL AND METHODS

Cell culture

The tumor cell line used in this study, MTLn3, is a highly invasive and metastatic clone derived from the 13762NF rat mammary adenocarcinoma and was provided by Dr. A. Neri (Hoffmann-La Roche, Nutley, NJ). Tumor cells were grown in Dulbecco's modified Eagle's (DME) culture medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2. The cells were subcultured at subconfluency by harvesting with 0.25% trypsin–0.02% EDTA.

Growth factor and antibodies

Recombinant human HGF was a gift of Dr. T. Nakamura (Osaka University, Japan). Monoclonal antibodies (MAbs) raised in hamster against rat α1 (HA31/8), α2 (HA1/29) and β1 (HA2/11) integrin subunits were provided by Dr. D.L. Mendrick (Human Genomic Systems, Boston, MA). Rabbit polyclonal anti-human α3 antiserum was purchased from Chemicon (Temecula, CA). Mouse MAb to rat α6 integrin was purchased from Serotec (Oxford, UK). Mouse mAb to rat α3 integrin (Ralph 3-1) was kindly provided by Dr. L. Reichardt (UCSF, San Francisco, CA). Rabbit polyclonal anti-human α5 was kindly given by Dr. P. Johnson (UCSF, San Francisco, CA). The anti-phosphotyrosine antibodies 4G10 and Py20 were purchased from Upstate Biotechnology (Lake Placid, NY) and Transduction Laboratories (Lexington, KY), respectively. Polyclonal antibody to mouse c-Met (C-16) was from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FAK mAb (2A7) and anti-FAK polyclonal antibody were from Upstate Biotechnology. Secondary antibodies anti-mouse and anti-rabbit IgG were purchased from Jackson Immunoresearch (West Grove, PA).

Migration assay

Cell migration was assayed using modified Boyden chambers (Neuroprobe, Bethesda, MD) with 8 μm porosity polyvinyl pyrrolidone-free (PVDF) polycarbonate filters (Nucleopore, Pleasanton, CA). The undersides of filters were pre-coated with adhesive proteins laminin 1 (derived from the Engelbreth-Holm-Swarm [EHS], tumor), fibronectin (Collaborative Research, Bedford, MA), or type I collagen (Celtrix, Palo Alto, CA), at 4°C overnight. The lower well of the chambers was filled with serum-free medium (control) containing HGF or anti-integrin antibodies. A 50 μl aliquot of cell suspension distributed to the upper chamber was incubated for 4 hr at 37°C. Cells migrating to the underside of the filter were counted under microscope (×400 magnification) after fixation and staining.

Adhesion assay

Microtiter plates (96-well) were pre-coated with the adhesive proteins laminin 1, type I collagen or fibronectin at different concentrations and incubated at 4°C overnight. Wells were blocked with 1% BSA for 45 min at 37°C. The cells (passages 5-20) were cultured in 2% serum-containing medium for 24 hr, harvested at about 70% confluency, resuspended in serum-free DME medium supplemented with 0.1% BSA and distributed to wells (8×10^4/well). The cells were incubated at 37°C in a 5% CO_2 atmosphere for 15 min with or without HGF (2.5–20 ng/ml). Nonadherent cells were removed by washing the plate 3 times with 0.1% BSA-containing medium; the adhering cells were fixed with 2% formaldehyde and stained with 2% crystal violet. After solubilization with SDS, the absorbance was measured at 562 nm on an ELISA plate reader. In some experiments, integrin function-perturbing antibodies were incubated with the cell suspension and held on ice for 15 min before the cells were distributed to the ECM protein-coated wells. The cells were incubated at 37°C for 30 min, and adhesion was evaluated by measuring the absorbance as described above.

Surface biotinylation of MTLn3 cells

Briefly, cell monolayers grown to confluency in 100 mm tissue culture dishes were washed twice with warm PBS, after which 2.5 ml of PBS containing 1 mg/ml of sulfo-NHS-biotin (Pierce, Rockford, IL) was added to each dish. Cells were lysed by scraping into ice-cold lysis buffer (2% Nonidet P-40 in 0.1 M Tris) with a cocktail of protease inhibitors. The extracts were immunoprecipitated with different antibodies to various integrin subunits coupled with protein A- or anti-mouse IgG–agarose and analyzed by SDS-PAGE. The blots were visualized with enhanced chemiluminescence reagent (ECL; Amersham, Arlington Heights, IL).

Detection of tyrosine-phosphorylated proteins in cell extracts

MTLn3 cells were cultured in 2% serum-containing medium for 24 hr and treated with 1.25–20 ng/ml of HGF for 10 min; then they were lysed in ice-cold RIPA lysis buffer containing 1% Nonidet P-40 and 1 mM Na_3VO_4. Cell lysates containing the same amount of protein were analyzed by SDS-PAGE in 7.5% acrylamide gels in non-reducing and reducing conditions, blotted on Immobilon-P membranes (Millipore, Bedford, MA), and probed with 1 μg/ml of anti-phosphotyrosine antibody 4G10 or PY20. Immunoreactive bands were visualized by horseradish peroxidase–conjugated secondary antibody (Amersham) followed by the ECL detection system.

In some experiments cells were untreated or treated with HGF in suspension, after being harvested with trypsin/EDTA from cultures in 2% serum-containing medium. They were then lysed and analyzed by Western blotting with anti-phosphotyrosine antibody, as described above.

In other experiments cells were harvested as above, distributed without or with HGF to culture dishes pre-coated with laminin 1, type I collagen or fibronectin, and lysed after 15 min. Tyrosine phosphorylation analysis was then performed.

Immunoprecipitation and immunoblotting analysis

MTLn3 cells were cultured in 2% serum-containing medium for 24 hr, treated with 10 or 20 ng/ml of HGF for 10 min, and lysed in RIPA lysis buffer. Equal amounts of protein were immunoprecipitated with anti–c-Met polyclonal antibody (C-16) coupled to protein A beads, or with anti-FAK antibody, either MAb 2A7 coupled to anti-mouse IgG–agarose beads or rabbit polyclonal antibody coupled to protein A. In other experiments, 24 hr 2% serum-cultured cells were harvested and treated with HGF while adhering on ECM protein-coated dishes or while suspended, then lysed as above and immunoprecipitated with anti-FAK antibody. Immunoprecipitated samples were analyzed by SDS-PAGE on 7.5% acrylamide gels and transferred to PVDF membrane. Blots were blocked with a 3% BSA-0.05% Tween-TBS solution and probed with antibody to c-Met, or with anti-FAK antibodies or anti-phosphotyrosine MAb (4G10 or PY20). The immunoreactive bands were detected with the ECL detection system.

FAK immunostaining of cells

MTLn3 cells were seeded on uncoated coverslips in 2% serum-containing medium for 24 hr. Then they were treated with HGF (10 ng/ml) or untreated for 10 min and stained with anti-FAK polyclonal antibody followed by FITC-labeled secondary antibody. Immunostaining was determined by observing microscopically the fluorescence intensity.

RESULTS

HGF induces scatter response and motility

MTLn3 cells normally form tightly clustered epithelial colonies. However, following treatment with HGF, the cells were induced to rapidly scatter. The originally epithelial cells were transformed into migratory fibroblast-like cells in which cell-cell contacts were disrupted (Fig. 1).

We further examined the induction of motility by HGF in MTLn3 cells using the modified Boyden chamber assay. HGF rapidly stimulated the motility of cells on several different ECM ligands, including laminin 1, type I collagen and fibronectin (Fig. 2*a*). Interestingly, the greatest stimulation of migration by HGF

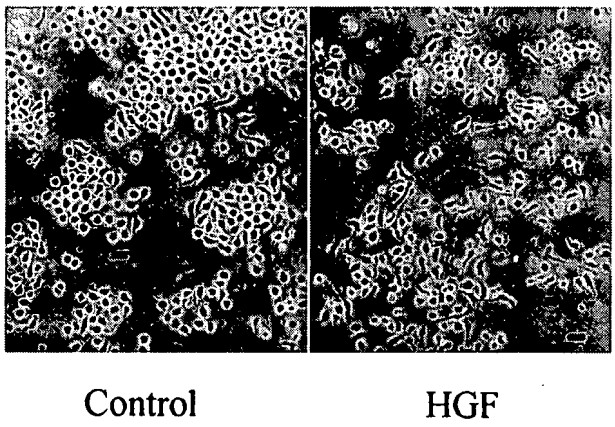

FIGURE 1 – HGF-induced scattering of MTLn3 carcinoma cells. Cells were incubated in control medium or treated with HGF at 10 ng/ml for 5 hr. The normally compact epithelioid colonies of tumor cells have dispersed as fibroblastic cells in response to HGF. Scale bar = 50 μm.

was observed on laminin 1-coated substrates. This motogenic effect was dose dependent, as shown for cell migration on laminin in the presence of different concentrations of HGF (Fig. 2*b*). Even low levels of HGF (2.5–5 ng/ml) induced locomotion, which approached a plateau at 20 ng/ml.

HGF stimulates adhesion of MTLn3 cells to ECM proteins

Since motility requires that cells first become attached, we also determined the adhesive abilities of MTLn3 cells in the presence of HGF. HGF's effect on adhesion paralleled its effect on cell motility. Adherence of cells treated with HGF on substrates coated with laminin, type I collagen, or fibronectin was 2- to 3-fold greater than that of control cells within 15 min (Fig. 3).

HGF-stimulated cell adhesion to laminin 1 was dose dependent (Fig. 3*b*). The stimulatory effect of HGF was maximal at about 10 ng/ml and then plateaued. Interestingly, type I collagen was a highly efficient substrate for MTLn3 cell attachment compared with laminin 1 or fibronectin, since a lower amount of type I collagen than of fibronectin or laminin 1 was needed to support cell adhesion. Importantly, the highest level of enhanced cell adhesion induced by HGF was on laminin 1 substrates.

Analysis of integrin expression and function

To characterize the relative expression of integrins on the surface of MTLn3 cells, we performed surface biotinylation followed by immunoprecipitation of detergent cell lysates with specific MAb (Fig. 4). The results showed that MTLn3 cells had moderate amounts of α2, α3, α5, and α6 integrins that were associated with their β1 partner. Immunoprecipitation with anti-α1 antibody recovered little if any material, indicating negligible amounts of α1β1 expression in these cells. Anti-β4 antibody (clone 3E1) recovered a small amount of the α6β4 complex (not shown), with major polypeptides corresponding to the expected 180–200, 170, and 130 kDa β4 subunit and its proteolytically cleaved polypeptides and the 140 kDa α6 subunit under non-reducing conditions (Giancotti *et al.*, 1992). However, most of the α6 was associated with the β1 partner. Flow cytometry confirmed the relative expression of individual integrins for which suitable antibodies were available. For example, relative mean fluorescence intensities were 280.8 for β1, 4.8 for α1 and 69.9 for α2 subunits.

Inhibition of cell adhesion by integrin-blocking antibodies

We tested different integrin function-blocking antibodies in adhesion assays. As expected, the anti-α1 (HA31/8) integrin-blocking antibody failed to have an inhibitory effect, since this

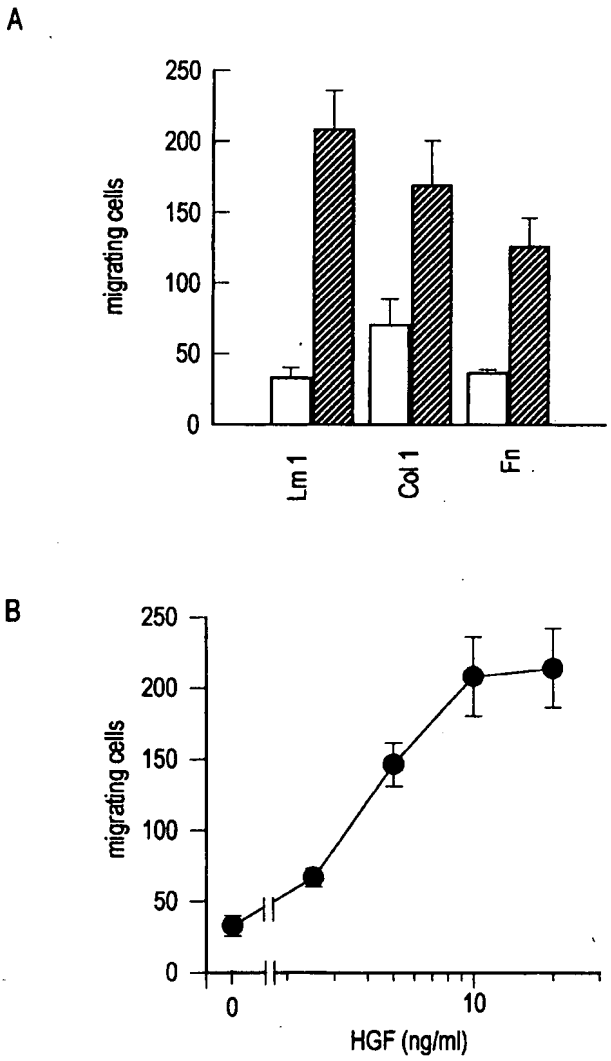

FIGURE 2 – Effect of HGF on MTLn3 cell migration on ECM ligands. (*a*) Tumor cells were placed in the upper wells of modified Boyden chambers containing filters coated on the underside with laminin 1, type I collagen or fibronectin. HGF (20 ng/ml) was added in the lower chamber. After 4 hr of incubation, the number of cells that had migrated to the underside of the membrane was counted in 4 randomly chosen fields (magnification ×400). Results are means of 6 different replicates; bars represent S.D. (*b*) Migration on laminin 1-coated filters (5 μg/ml) in the presence of HGF (2.5–20 ng/ml). This result, a representative experiment with means of 6 replicates, showed a dose-dependent effect of HGF on cell migration on laminin.

integrin is not expressed by these cells (Fig. 5*a*). However, anti-α2 (HA1/29), anti-rat α3 (Ralph 3–1) and anti-β1 (HA2/11) integrin function-perturbing antibodies inhibited cell adhesion to and spreading on laminin by about 30, 50 and 90%, respectively. Moreover, cell adhesion to laminin 1 could be nearly completely blocked (80% inhibition) by the combination of anti-α2 and anti-α3 MAbs (Fig. 5*a*). Although MTLn3 cells express significant levels of α6 integrin, the lack of anti-rat α6 blocking MAb precluded the possibility of assessing this integrin's function in adhesion.

On type I collagen substrates, we found that anti-α2 and anti-β1 integrin function-perturbing MAbs almost completely inhibited cell adhesion to this substrate; in contrast, the anti-α1 and -α3

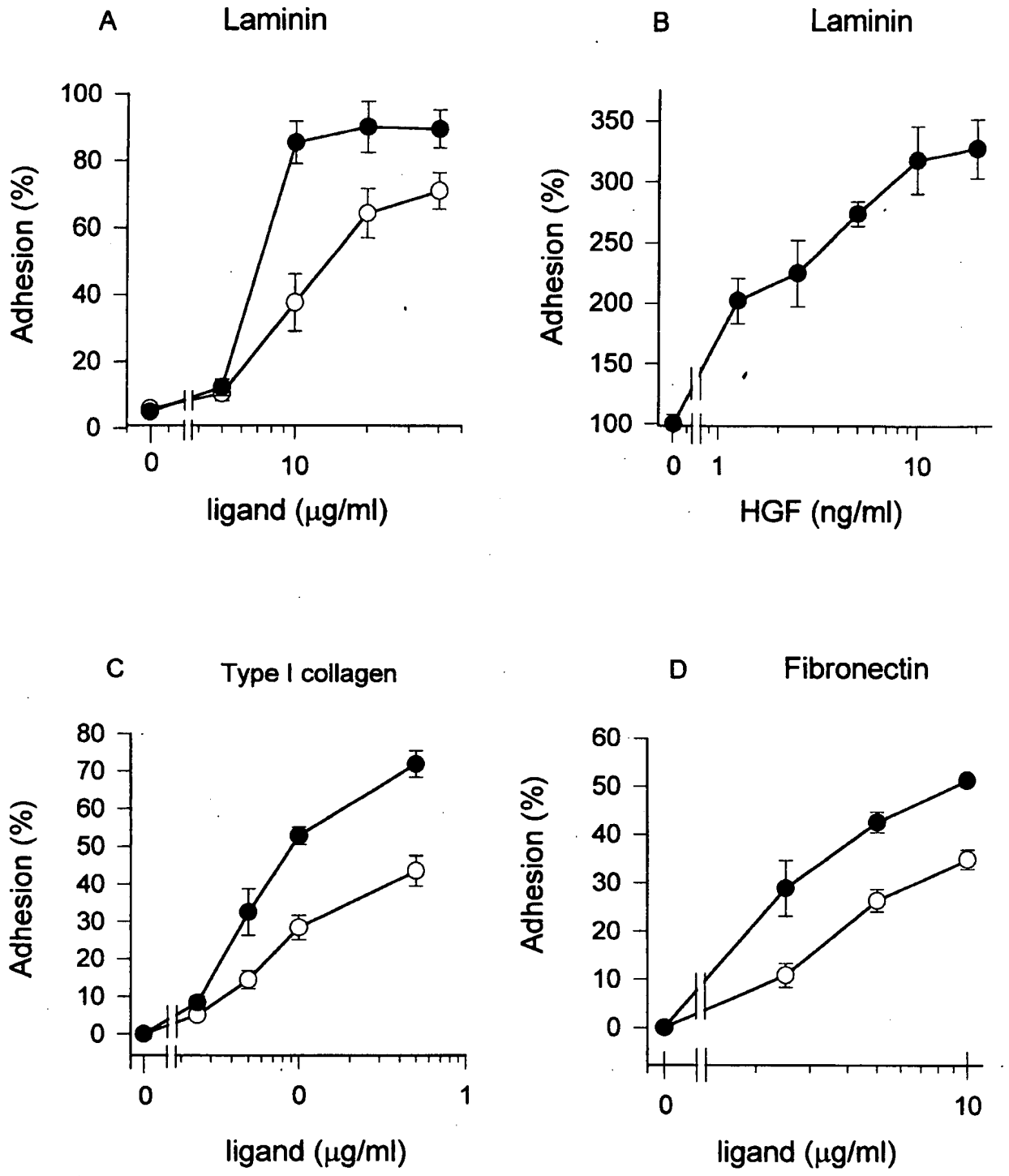

FIGURE 3 – Stimulatory effect of HGF on MTLn3 cell adhesion to ECM proteins. Tumor cells were seeded on 96-well dishes coated with different concentrations of (*a,b*) laminin 1, (*c*) type I collagen, and (*d*) fibronectin, without (open circle) or with (closed circle) HGF (20 ng/ml). Cells were stimulated by HGF to adhere to all 3 ECM proteins within 15 min. Data, shown as means of 4 replicates, are expressed as percent adhesion and are representative of at least five individual experiments. Bars represent S.D. (*a*) Dose-response of HGF-stimulated cell adhesion to laminin 1- coated dishes.

integrin antibodies failed to produce an inhibitory effect (Fig. 5*b*). This indicated that MTLn3 cells primarily use $\alpha 2\beta 1$ integrin to adhere to type I collagen.

Inhibition of cell migration by integrin-blocking antibodies

Next, we performed migration assays of MTLn3 cells on laminin 1 and type I collagen substrates in the presence and absence of

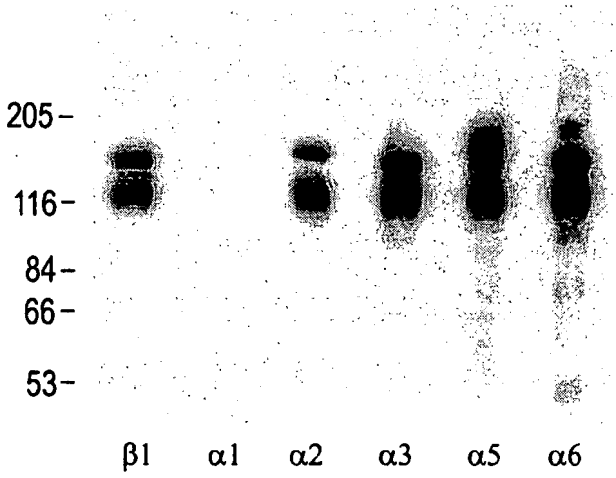

FIGURE 4 – Analysis of integrin heterodimers. MTLn3 cells were biotinylated and then extracted for immunoprecipitation with anti-integrin antibodies: anti-β1 (lane 1), anti-α1 (lane 2), anti-α2 (lane 3), anti-α3 (lane 4), anti-α5 (lane 5) and anti-α6 (lane 6). Samples were analyzed by SDS-PAGE in 7.5% polyacrylamide gels under reducing conditions, and visualized by chemiluminescence. The positions of molecular mass markers in kDa are indicated.

available integrin inhibitory antibodies. We found that α2 integrin and α3 integrin function-blocking antibodies effectively blocked cell migration on laminin 1; in the presence of anti-β1 antibody, the number of migrating cells decreased nearly 80% (Fig. 6*a*). This result indicated that both α2β1 and α3β1 integrin complexes play a role in MTLn3 cell migration on laminin as well as in adhesion.

As expected, MTLn3 cell migration on type I collagen was not prevented by anti-α1 integrin antibody but was significantly inhibited by anti-α2 and anti-β1 antibodies (Fig. 6*b*). This suggested that the α2β1 integrin plays the major role in mediating motility as well as adhesion of these cells on type I collagen.

Tyrosine phosphorylation induced by HGF

Since the rapid modulation of adhesion of cells treated with a growth factor is often accompanied by changes in protein tyrosine phosphorylation, we wondered whether the stimulatory effect of HGF on adhesion of MTLn3 cells might involve activation of any cellular protein downstream from the tyrosine kinase receptor c-Met. Using anti-phosphotyrosine antibody, we performed immunoblot analysis of cell lysates prepared from 24 hr 2% serum-containing cultures of MTLn3 cell cultures treated with HGF or untreated.

HGF increased tyrosine phosphorylation of a number of proteins in MTLn3 cells in a dose-dependent manner (Fig. 7*a*). In particular, enhanced tyrosine phosphorylation of 2 polypeptides of 145 and 125 kDa was detected in response to HGF. We next assessed whether the 145-kDa protein was c-Met, using immunoprecipitation analysis. Cells were stimulated with HGF, solubilized and immunoprecipitated with anti-c-Met antibody (C16). Analysis of the immunoprecipitates by immunoblotting with anti-phosphotyrosine antibody PY20 verified that the 145 kDa polypeptide β-chain of c-Met was activated by HGF (Fig. 7*b*).

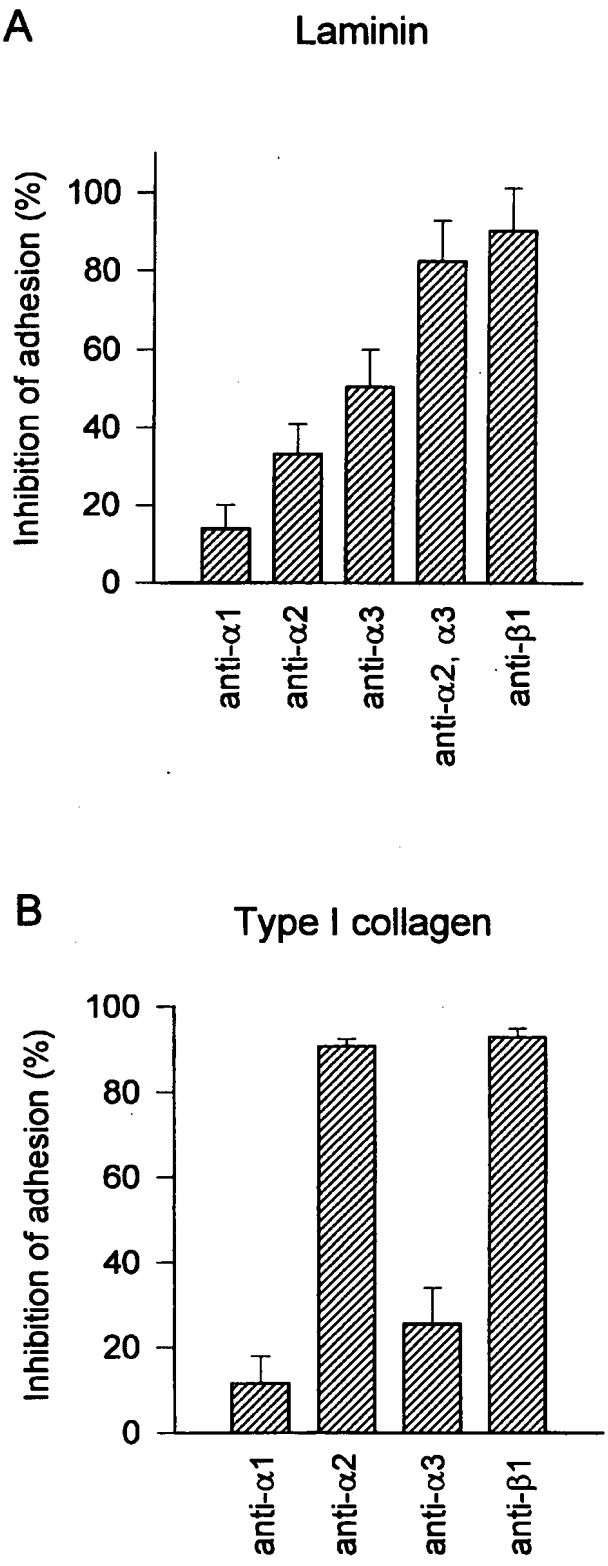

FIGURE 5 – Effects of anti-integrin antibodies on MTLn3 cell adhesion to (*a*) laminin 1 (10 μg/ml) and to (*b*) type I collagen (0.5 μg/ml). Tumor cells were preincubated with antibodies to α1 (clone HA31/8, 10 μg/ml), α2 (clone HA1/29, 10 μg/ml), α3 (hybridoma supernatant), and β1 (clone HA2/11, 10 μg/ml) and held on ice for 15 min. They were then distributed to 96-well plates pre-coated with the ECM proteins and incubated at 37°C for 30 min. Adhesion assays were performed as indicated in Material and Methods. Results are shown as percent inhibition of control adhesion, assessed in the absence of antibodies; bars represent S.D.

Identification of FAK

To determine whether the 125 kDa tyrosine-phosphorylated protein was FAK, we immunoprecipitated cell lysates with anti-FAK polyclonal or monoclonal antibody, followed by immunoblotting with anti-phosphotyrosine antibody. We detected a significant and specific increase of tyrosine-phosphorylated FAK in MTLn3 cells treated with 10 ng/ml of HGF compared with control (Fig. 7*d*, right panel).

Identification of a FRNK-like protein

Moreover, a lower band corresponding to about a 40 kDa protein was detected in the FAK blot from cell lysates immunoprecipitated with anti-FAK for protein loading control (Fig. 7*d*, left panel). This lower band was also detected in the FAK immunoblot from whole cell lysate (Fig. 7*c*, left panel). Moreover, it could not be clearly detected in the blot from lysates immunoprecipitated with anti-FAK and probed with anti-phosphotyrosine antibody, neither in control nor in HGF-treated cells (Fig. 7*d*, right panel), as well as in the anti-phosphotyrosine blot from the whole cell lysate (Fig. 7*c*, right panel). On the basis of these observations, we think that the 40 kDa band is likely to correspond to a FRNK-like molecule, the non-catalytic COOH-terminal domain of FAK. Indeed, FRNK does not contain tyrosine residues, and this explains the lack of its detection in the anti-phosphotyrosine blot.

Enhanced focal adhesion formation by HGF

We next determined whether HGF treatment resulted in recruitment of FAK to focal adhesions. MTLn3 cells were seeded on uncoated coverslips and cultured in 2% serum for 24 hr, then treated with HGF or untreated for 10 min and stained with anti-FAK antibody followed by FITC-conjugated secondary antibody. In response to HGF, cells showed extensive formation of new focal adhesions, as indicated by greatly increased staining for FAK at the cell periphery (Fig. 8). These FAK-positive focal contacts also stained for vinculin and α-actinin (not shown).

Dependence of HGF-induced FAK activation on cell adhesion

To assess whether HGF-induced activation of FAK requires concurrent and specific integrin engagement, we studied tyrosine phosphorylation of FAK immunoprecipitated from MTLn3 cells attached to type I collagen, fibronectin, or laminin 1 substrates (Fig. 9). On all substrates, HGF-induced stimulation of FAK autoactivation was detected. The relative increase in FAK tyrosine phosphorylation following HGF treatment compared to control cultures varied depending on the substrate ligand. On type I collagen substrates, a strong HGF-induced stimulation of tyrosine phosphorylation was observed. On fibronectin substrates, the HGF-induced stimulation of FAK tyrosine phosphorylation was less than that produced on type I collagen substrates, owing to the somewhat higher level of baseline phosphorylation. For laminin 1 substrates, an increase in FAK autophosphorylation was observed but was less intense than for the other 2 ligands. Such differences in the control level of FAK activation and HGF-induced stimulation may be related to differences in initial adhesion rates to each substrate.

Next, to determine whether cell attachment was required for HGF activation of FAK, we immunoprecipitated protein lysates from suspended and attached cells with anti-FAK antibody, followed by immunoblotting with anti-phosphotyrosine antibody. No HGF-induced increase in tyrosine phosphorylation of FAK was detected in suspended MTLn3 cells as compared with control (Fig. 10). In contrast, attached cells showed the expected increase in FAK tyrosine phosphorylation when stimulated with HGF (Fig. 10).

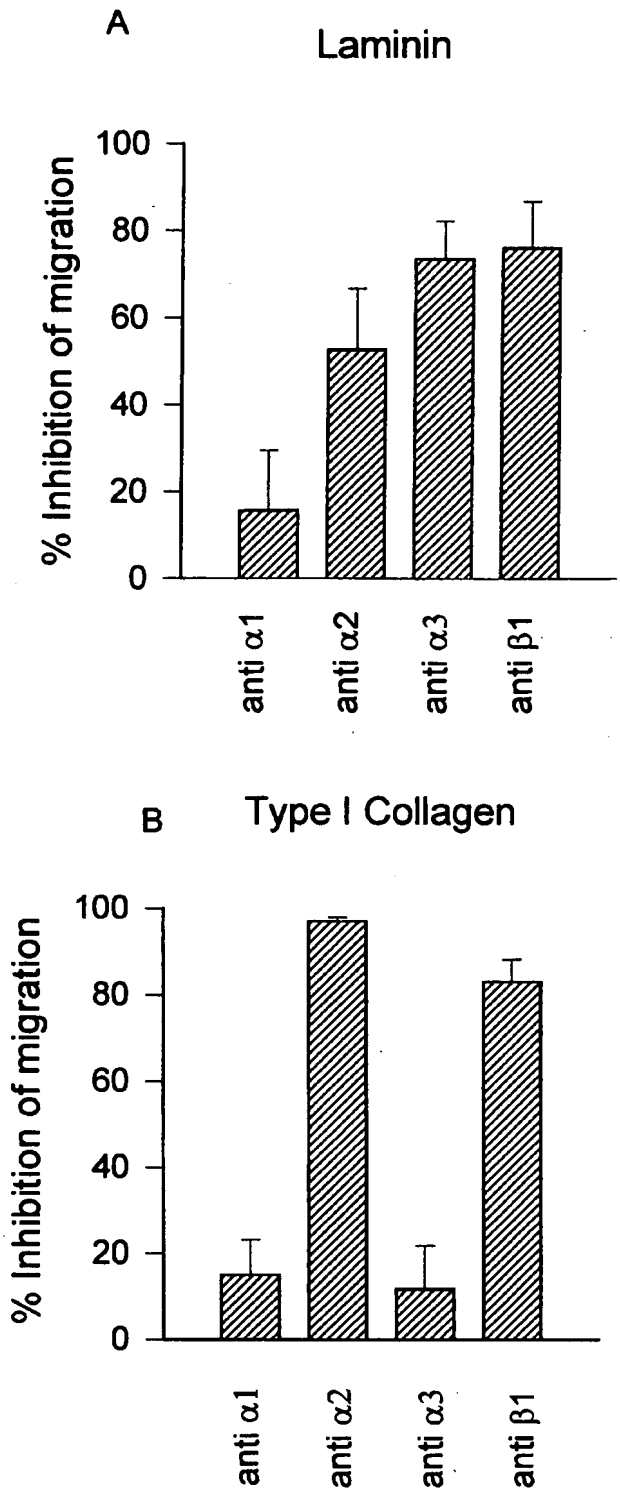

FIGURE 6 – Effects of anti-integrin antibodies on MTLn3 cell migration on (*a*) laminin 1 and (*b*) type I collagen. Different integrin function-blocking antibodies (used at the same concentrations as in adhesion inhibition assays) were added to the lower wells of Boyden chambers, and MTLn3 cells were placed in the upper chamber wells. Undersides of filters were coated with laminin 1 or type 1 collagen (10 μg/ml). Results are shown as percent inhibition of control migration within 4 hr in the presence of anti-α1, -α2, -α3 and -β1 integrin MAbs. Bars represent S.D.

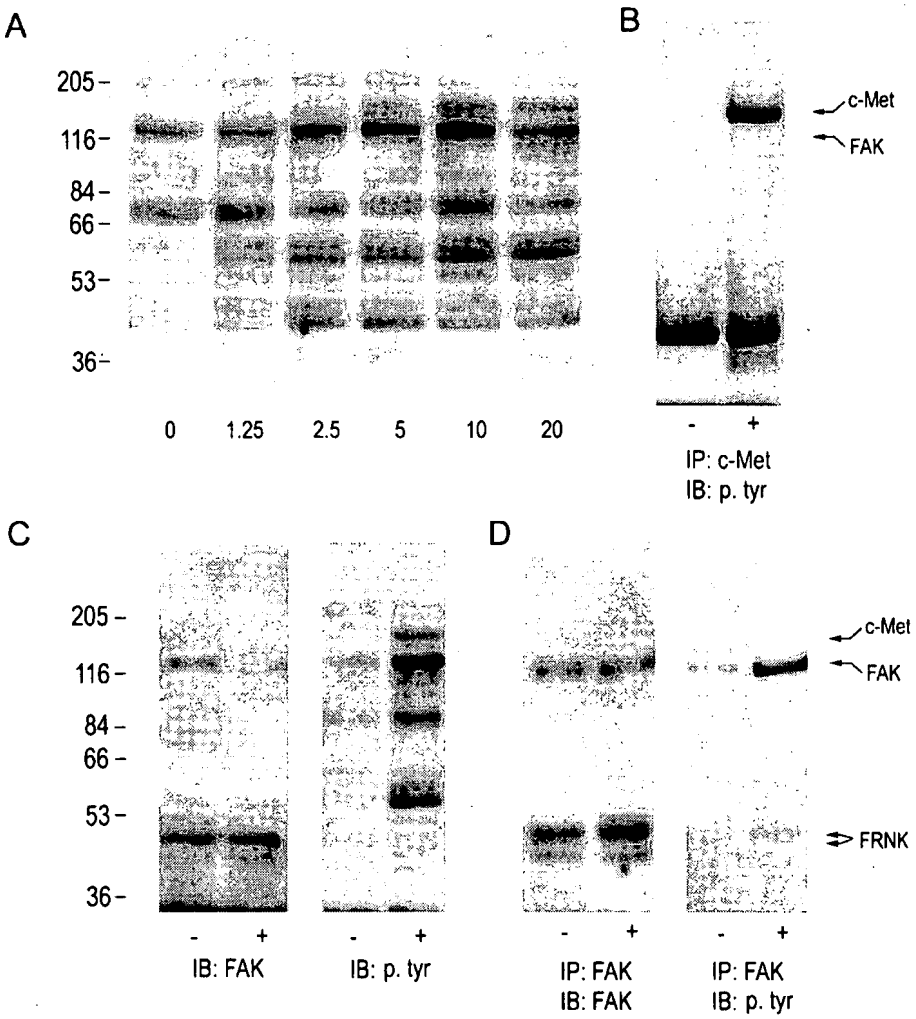

FIGURE 7 – Analysis of tyrosine phosphorylation. (*a*) Dose-dependent protein tyrosine phosphorylation profile in response to HGF. MTLn3 cell cultures in 2% serum-containing medium were untreated (0) or treated with HGF (1.25–20 ng/ml) for 10 min before lysis. Cell lysates containing the same amount of protein were analyzed by SDS-PAGE in 7.5% polyacrylamide gels under reducing conditions, and immunoblotted with anti-phosphotyrosine antibody. Lysates from MTLn3 cells untreated (−) or treated with (+) 10 ng/ml HGF were immunoblotted with anti-FAK antibody (*c*, left panel) or anti-phosphotyrosine antibody (*c*, right panel). Cell extracts, prepared as above, were immunoprecipitated with anti-c-Met (*b*) or anti-FAK antibody (*d*, right panel) and immunoblotted with anti-phosphotyrosine antibody. The same blot from FAK immunoprecipitation and analysis for phosphotyrosine was stripped and re-probed with anti-FAK antibody for protein loading control (*d*, left panel).

DISCUSSION

HGF and the c-Met tyrosine kinase receptor have been implicated in the development and growth of breast carcinoma (Tuck *et al.*, 1996). HGF promotes invasion of breast carcinoma cells, suggesting that this growth factor and its receptor are involved in tumor dissemination (Beviglia *et al.*, 1997). High levels of HGF/scatter factor (SF) in primary human breast carcinomas have been found to correlate with aggressive tumors and poor survival (Yamashita *et al.*, 1994). Importantly, c-Met is frequently overexpressed in human breast carcinoma and may be related to an invasive phenotype (Tuck *et al.*, 1996; Beviglia *et al.*, 1997). Taken together, there is substantial evidence to suggest that HGF/SF and the c-Met receptor play an important role in tumor progression in human breast cancer.

In the current study, we investigated the effect of HGF on the adhesive properties of MTLn3 rat breast carcinoma cells. In addition to inducing cell scattering and stimulating cell migration on ECM proteins, HGF rapidly stimulated MTLn3 cell adhesion to the ECM ligands laminin 1, type I collagen and fibronectin. Weimar *et al.* (1997) reported also that HGF induced an increase in lymphoma cell adhesion to fibronectin and type I collagen. However, in the Weimar study HGF failed to stimulate lymphoma cell adhesion to laminin, whereas in our study the effect of HGF appeared to be less specific. Moreover, the highest level of enhanced cell adhesion induced by HGF was on laminin 1 substrates. Therefore the differences in the HGF effect may be dependent, in part, on the tumor cell type.

Integrins appear to be a primary mechanism by which tumor cells interact with the ECM leading to invasion and metastasis (Stetler-Stevenson *et al.*, 1993). We have found that the HGF-induced increases in MTLn3 cell adhesion and migration were mediated by β1 integrins; α2β1 and α3β1 mediated most of the cell adhesion and migration on laminin 1 and showed a synergistic effect, and α2β1 was the major receptor for type I collagen.

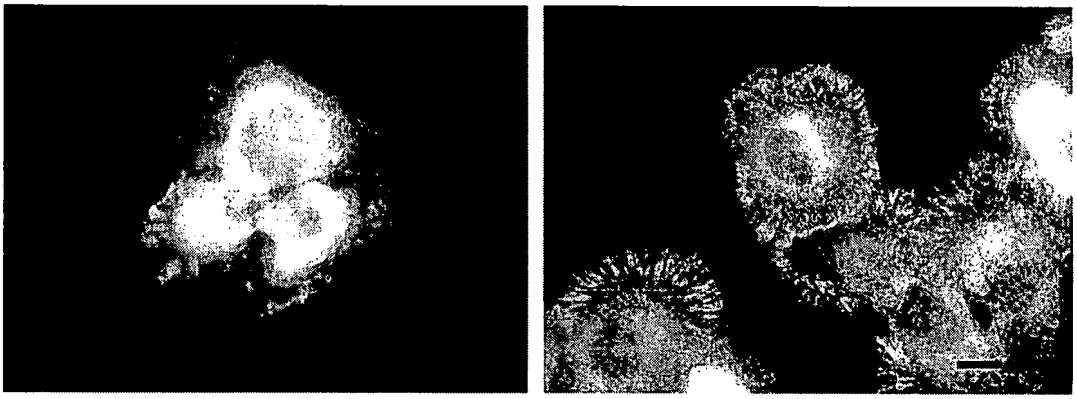

FIGURE 8 – FAK immunostaining. Tumor cells were seeded on uncoated coverslips in 2% serum-containing medium for 24 hr, treated with HGF or untreated for 10 min, and stained for FAK. Cells showed extensive lamellipodia protrusions in response to HGF, likely due to formation of new focal adhesions, as indicated by greatly increased staining for FAK at the cell periphery. Scale bar = 30 μm.

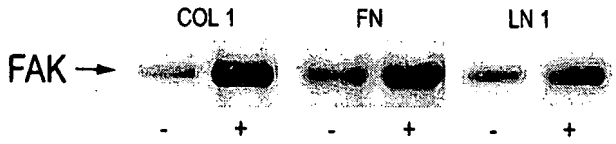

FIGURE 9 – Dependence of HGF-induced tyrosine phosphorylation of FAK on cell adhesion. MTLn3 cells were harvested and allowed to adhere to plastic dishes precoated with type I collagen (0.5 μg/ml), fibronectin (5 μg/ml), or laminin 1 (10 μg/ml) without (−) or with (+) HGF (10 ng/ml) at 37°C for 15 min. Cell extracts were immunoprecipitated with anti-FAK polyclonal antibody and immunoblotted with anti-phosphotyrosine antibody. HGF stimulated tyrosine phosphorylation of FAK in HGF-treated cells adherent on all substrates.

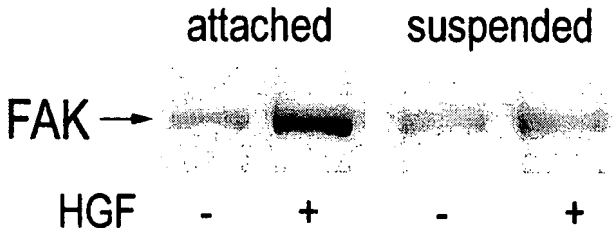

FIGURE 10 – Tyrosine phosphorylation analysis in suspended MTLn3 cells. Adherent or freshly harvested and suspended cells were untreated (−) or treated with (+) HGF (10 ng/ml) for 10 min at 37°C and lysed. Lysates containing the same amount of protein were subjected to immunoprecipitation analysis with anti-FAK antibody and immunoblotted with anti-phosphotyrosine MAb. No change in the low level of tyrosine-phosphorylated FAK was detected in suspended cells without or with HGF; however, an increase in tyrosine-phosphorylated FAK was detected in HGF-treated adherent cells as compared with control cells.

Because of the unavailability of function-blocking antibody, we were unable to determine the contribution of α6 integrin to MTLn3 cell adhesive interactions with laminin. However, since the combination of blocking MAb to α2 and α3 integrins effectively inhibited adhesion to laminin, we conclude that α6 integrin is not active in the MTLn3 cells.

Moreover, in our study, HGF induced formation of lamellipodia protrusions and new focal adhesions and possibly rapid recruitment of FAK to focal contacts. In correlation with these events induced by HGF, we observed a specific and significant increase in tyrosine phosphorylation of FAK. Our results suggest that FAK may play a role in the HGF stimulation of focal adhesions of MTLn3 cells. Also, the HGF-induced stimulation of FAK tyrosine phosphorylation in MTLn3 cells was independent of the type of integrin receptors engaged with ECM. Although small differences were observed in the control level of FAK activation and HGF-induced stimulation, these were due to differences in cell adhesion rate to each substrate.

FAK tyrosine phosphorylation occurs during formation of focal adhesions and spreading on ECM (Burridge *et al.*, 1992). Moreover, FAK is involved in the growth factor signaling cascade. Our results provide additional evidence for the involvement of FAK in such signaling pathways, and also provide evidence for the activation of FAK by HGF in breast carcinoma cells. Although others have shown that EGF stimulates MTLn3 cell adhesion and spreading (Lichener *et al.*, 1993), an increase in tyrosine phosphorylation of FAK has not been reported. Possibly, FAK could also be involved in the effects of EGF and have an important role in the metastatic dissemination of breast carcinoma.

In our study, the integrin/ECM and the HGF/c-Met signaling pathways appeared to cooperate to induce FAK activation in an adhesion-dependent manner. Indeed, HGF failed to stimulate FAK tyrosine phosphorylation in MTLn3 cells in suspension, which suggests that integrin adhesive function is required for HGF to induce activation of FAK, and may exclude the possibility of different mechanisms by which the receptor c-Met and integrins stimulate FAK. After completion of our work, Chen *et al.* (1998) reported that stimulation of tyrosine phosphorylation of FAK by HGF in human embryonic kidney 293 cells is independent of cell adhesion and does not involve integrin signaling. Therefore, in contrast with these results, our findings are important in that they provide evidence of HGF-induced FAK tyrosine phosphorylation as a consequence of integrin engagement in the breast carcinoma cells examined.

Many studies have focused on HGF-induced scattering activity and subsequent dissociation of cells and stimulated locomotion. (Matsumoto *et al.*, 1994; Jiang *et al.*, 1999). Tumor cell adhesion to substrata is a prerequisite before cells migrate and invade. Therefore, our results suggest that the effect of HGF on migration is a secondary response. Cell adhesion appears to involve assembly of focal adhesions first, whereas motility requires their disassembly. Focal contacts contain an assembly of kinases (Yamada and Geiger, 1997) (*e.g.*, Src, Rho-family GTPase, PI-3 kinase) and cytoskeletal

proteins (*e.g.*, vinculin, actinin, paxillin), and FAK interacts with several cytoskeletal proteins at these sites. We previously reported that in oral squamous carcinoma cells, HGF induced an increase in tyrosine phosphorylation of FAK at focal adhesions for up to 30 min, followed by spreading; after this time, FAK tyrosine phosphorylation and its localization in focal adhesions decreased in correlation with migration of cells (Matsumoto *et al.*, 1994). In the present study, we also found a time-dependent increase in protein tyrosine phosphorylation: MTLn3 cells showed a maximum increase at 10 min, persisting to 30 min, and a decrease thereafter (data not shown). Therefore our results, in accordance with those of Matsumoto *et al.* (1994), suggest that after accumulation and activation of both cytoskeletal proteins and FAK in the focal contacts during cell adhesion and spreading, attenuation of activation events takes place together with disassembly of focal adhesions and cell motility. However, the mechanism by which HGF promotes MTLn3 cell migration other than cell adhesion is less clear and the precise role of FAK in cell motility remains to be better elucidated, despite the extensive evidence shown for the activation of FAK during cell adhesion and spreading.

Ilic *et al.* (1995) showed that FAK-null embryonic mouse fibroblasts did form focal adhesions and had a high level of tyrosine phosphorylation of several cytoskeletal proteins; however, these cells had impaired motility. This finding suggests that FAK is important for cell motility and it might act as a regulatory protein that also coordinates disassembly of focal contacts, as occurs during cell motility. However, in our study we found that FAK tyrosine phosphorylation decreases after 30 min of HGF treatment and that MTLn3 cells in the presence of HGF migrate more than untreated cells within 4 hr. Therefore, there must be some mechanism by which HGF stimulates cell locomotion, and this effect does not appear to be due directly to tyrosine phosphorylation of FAK that decreases with time, whereas its increase is required for stimulation of cell adhesion. Richardson and Parsons (1996) have noted that tyrosine phosphorylation of FAK is important in regulating formation of new focal adhesions, which is consistent with our observations showing formation of lamellipodia extensions in continually adherent MTLn3 cells treated with HGF for 10 min. Richardson and Parsons (1996) have also reported that FRNK overexpression in chicken fibroblasts determines a delayed cell spreading on fibronectin. In addition, a GST–C-terminus fusion protein containing the FAK focal adhesion targeting sequence induces decrease of motility and proliferation in endothelial cells (Gilmore and Romer, 1996). On the basis of our results, showing that a FRNK–like protein is expressed by MTLn3 cells and perhaps is upregulated by HGF treatment, it is reasonable to assume that FRNK might explain in part the HGF-induced motility of these cells. Indeed, it is possible that the FRNK-like molecule in our system might determine decrease of FAK tyrosine phosphorylation and relocation of FAK from focal adhesions to the inside of cells that results in a motile cellular phenotype. In conclusion, both FAK and FRNK might control the adhesive properties of MTLn3 cells, determining, respectively, assembly of focal adhesions first and their disassembly later together with cell locomotion.

All existing studies have reported the effect of FRNK or the C-terminus of FAK in normal cells; there is a lack of experimental evidence for its role in tumor cells. Interestingly, when the carboxy-terminal domain of FAK corresponding to FRNK is exogeneously expressed into BT474 human breast carcinoma cells, it causes loss of adhesion and viability, decrease of FAK activation and inhibition of cell growth (Xu *et al.*, 1998). Since we also observed HGF-induced inhibition of MTLn3 cell growth by 50% within 4 days (not shown), the interpretation could be that a FRNK-like molecule we detected might be responsible, at least in part, for the HGF-induced inhibition of MTLn3 cell growth.

Autophosphorylation of c-Met induced by binding of its ligand, HGF, initiates a cascade of intracellular events involving activation of multiple downstream signaling pathways. Several protein kinases have been shown to be activated by and associated with tyrosine-phosphorylated c-Met, such as Grb, Src, MAP kinase, Ras, and PI3-kinase (Jiang *et al.*, 1999). Our results indicate that FAK, activated by HGF, may associate with c-Met and play an important role in the HGF/c-Met signaling cascade in MTLn3 cells. Since FAK lacks SH2 domains and needs adaptor proteins to interact with phosphorylated tyrosine residues, direct association of FAK with activated c-Met is unlikely. PI3-kinase and Src are possible candidates for mediating association of FAK with c-Met in MTLn3 cells.

Stimulation of FAK has not been studied in tumor cells as much as it has in fibroblasts (Hatai *et al.*, 1994). However, some studies have indicated overexpression of FAK in invasive tumors (Owens *et al.*, 1995). Also, a constitutive activation of FAK is often observed in tumors. Interestingly, MTLn3 tumor cells appear to differ in that they have low levels of expression and activation of FAK, in contrast to HSC-3 oral carcinoma cells previously studied. This observation suggests that the expression of a FRNK-like protein in MTLn3 cells could affect expression as well activation of FAK that remains low in the untreated cells and greatly increases by treatment with HGF. Therefore, gaining an understanding of the pattern of activation and function of FAK, and perhaps FRNK, in tumor progression to an invasive phenotype appears worthwhile.

ACKNOWLEDGEMENTS

The authors gratefully knowledge Dr. Toshikazu Nakamura (Osaka University) for human recombinant HGF and also thank Ms. Evangeline Leash for editing the manuscript. LB is a recipient of an award fellowship from the American Italian Cancer Foundation (grant 94–102).

REFERENCES

ABEDI, H. and ZACHARY, I., Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. *J. biol. Chem.*, **272**, 15442–15451 (1997).

BEVIGLIA, L., MATSUMOTO, K., LIN, C.S., ZIOBER, B. and KRAMER, R.M., Expression of the c-Met/HGF receptor in human breast carcinoma: correlation with tumor progression. *Int. J. Cancer*, **74**, 301–309 (1997).

BURRIDGE, K., TURNER, C.E. and ROMER, L.H., Tyrosine phosphorylation of paxillin and pp125^{FAK} accompanies cell adhesion to extracellular matrix: a role in cytoskeletal assembly. *J. cell Biol.*, **119**, 893–903 (1992).

CHEN, H.C., CHAN, P.C., TANG, M.J., CHENG, C.H. and CHANG, T.J., Tyrosine phosphorylation of focal adhesion kinase stimulated by hepatocyte growth factor leads to mitogen-activated protein kinase activation. *J. biol. Chem.*, **273**, 25777–25782 (1998).

GIANCOTTI, F.G., STEPP, M.A., SUZUKI, S., ENGVALL, E., and RUOSLAHTI, E., Proteolytic processing of endogenous and recombinant beta 4 integrin subunit. *J. cell Biol.*, **118**, 951–959 (1992).

GILMORE, A.P., and ROMER, L.H., Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol. Biol. Cell.*, **7**, 1209–1224 (1996).

HATAI, M., HASHI, H., MOGI, A., SOGA, H., YOKOTA, J., and YAOI, Y., Stimulation of tyrosine- and serine-phosphorylation of focal adhesion kinase in mouse 3T3 cells by fibronectin and fibroblast growth factor. *FEBS Lett.*, **350**,113–116 (1994).

HYNES, R.O., Integrins: versatility, modulation and signaling in cell adhesion. *Cell*, **69**, 11–25 (1992).

ILIC, D., FURUTA, Y., KANAZAWA, S., TAKEDA, N., SOBUE, K., NAKATSUJI, N., NOMURA, S., FUJIMOTO, J., OKADA, M., YAMAMOTO, T. and ALZAWA, S., Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature (Lond.)*, **377**, 539–544 (1995).

JIANG, W., HISCOX, S., MATSUMOTO, K., and NAKAMURA, T., HGF/SF, its molecular, cellular, and clinical implications in cancer. *Crit. Rev. Oncol. Hematol.*, **29**, 209–248 (1999).

LICHENER, R.B., WIEDEMUTH, M., CHRISTINE, N.J., and SCHIRMACHER, V., Rapid effects of EGF on cytoskeletal structures and adhesive properties of highly metastatic rat mammary adenocarcinoma cells. *Clin. exp. Metastasis,* **11**, 113–125 (1993).

MATSUMOTO, K., MATSUMOTO K., NAKAMURA, T. and KRAMER, R.H., Hepatocyte growth factor/scatter factor induces tyrosine phosphorylation of focal adhesion kinase (p125FAK) and promotes migration and invasion by oral squamous carcinoma cells. *J. biol. Chem.*, **269**, 31807–31813 (1994).

OWENS, L.V., XU, L., CRAVEN, R.J., DENT, G.A., WEINER, T.M., KORNBERG, L., LIU, E.T., and CANCE, W.G., Overexpression of the focal adhesion kinase ($p125^{FAK}$) in invasive tumors. *Cancer Res.*, **55**, 2752–2755 (1995).

RICHARDSON, A., and PARSONS, T., A mechanism for regulation of the adhesion-associated protein tyrosine kinase $pp125^{FAK}$. *Nature (Lond.),* **380**, 358–340 (1996).

STETLER-STEVENSON, W.C., AZNAVOORIAN, S., and LIOTTA, L.A., Tumor cell interactions with the extracellular matrix during invasion and metastasis. *Ann. Rev. Cell Biol.*, **9**, 541–573 (1993).

TAJIMA, H., MATSUMOTO, K. and NAKAMURA, T., Hepatocyte growth factor has potent anti-proliferative activity in various tumor cell lines. *FEBS Lett.*, **291**, 229–232 (1991).

TUCK, A.B., PARK, M., STERN, E.E., BOAG, A. and ELLIOTT, B.E., Coexpression of hepatocyte growth factor and receptor (Met) in human breast carcinoma. *Amer. J. Pathol.*, **148**, 225–232 (1996).

WEIMAR, I.S., DE JONG, D., MULLER, E.J., NAKAMURA, T., VAN GORP, J.M., DE GAST, G.C. and GERRITSEN, W.R., Hepatocyte growth factor/scatter factor promotes adhesion of lymphoma cells to extracellular matrix via $\alpha4\beta1$ and $\alpha5\beta1$ integrins. *Blood,* **89**, 990–1000 (1997).

XU, L., YANG, X., CRAVEN, R.J., and CANCE, W.G., The COOH-terminal domain of the focal adhesion kinase induces loss of adhesion and cell death in human tumor cells. *Cell Growth Differentiation,* **9**, 999–1005 (1998).

YAMADA, K. and GEIGER, B., Molecular interactions in cell adhesion complexes. *Curr. Opinion cell Biol.*, **9**, 76–85 (1997).

YAMASHITA, J., OGAWA, M., YAMASHITA, S., NOMURA, K., KURAMOTO, M., SAISHOJI, T., and SHIN, S., Immunoreactive hepatocyte growth factor is a strong and independent predictor of recurrence and survival in human breast cancer. *Cancer Res.*, **54**, 1630–1633 (1994).

NPL___ ADONIS___ MIC___ BioT X Main__NO__Vol NO__ NOS___CKCite__Dup__Int SAS 4-2-07

RC 268.42.048

STIC-ILL

From: Davis, Minh-Tam
Sent: Monday, April 02, 2007 4:06 PM
To: STIC-ILL
Subject: Reprint request for 10/500297

1) Presence and tyrosine phosphorylation of c-met receptor in human sperm.
Herness E A; Naz R K
Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo 43614-5806, USA.
Journal of andrology (UNITED STATES) Sep-Oct 1999, 20 (5) p640-7, ISSN 0196-3635--Print Journal Code: 8106453
Contract/Grant No.: HD24425; HD; NICHD

2) HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells.
Beviglia L; Kramer R H
Department of Stomatology, University of California, San Francisco, San Francisco, CA, USA.
International journal of cancer. Journal international du cancer (UNITED STATES) Nov 26 1999, 83 (5) p640-9, ISSN 0020-7136--Print
Journal Code: 0042124

3) Title: OVEREXPRESSION OF THE C-MET HGF RECEPTOR GENE IN HUMAN THYROID CARCINOMAS (Abstract Available)
Author(s): DIRENZO MF; OLIVERO M; FERRO S; PRAT M; BONGARZONE I; PILOTTI S; BELFIORE A; COSTANTINO A; VIGNERI R; PIEROTTI MA; COMOGLIO PM
Corporate Source: UNIV TURIN,SCH MED,DEPT BIOMED SCI & ONCOL/I-10124 TURIN//ITALY/; NATL CANC INST,DEPT EXPTL ONCOL A/MILAN//ITALY/; NATL CANC INST,DEPT PATHOL/MILAN//ITALY/; UNIV CATANIA,SCH MED,DEPT ENDOCRINOL/I-95124 CATANIA//ITALY/
Journal: ONCOGENE, 1992, V7, N12 (DEC), P2549-2553
ISSN: 0950-9232

4) 268602 PMID: 9065649
Detection of MET oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell carcinomas.
Galeazzi E; Olivero M; Gervasio F C; De Stefani A; Valente G; Comoglio P M; Di Renzo M F; Cortesina G
Department of Clinical Physiopathology, University of Turin School of Medicine, Italy.
European archives of oto-rhino-laryngology - official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) - affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery (GERMANY) 1997, 254 Suppl 1 pS138-43, ISSN 0937-4477--Print Journal Code: 9002937

THANK YOU.

MINH TAM DAVIS
ART UNIT 1642, REMSEN 3A24, MB 3C18
272-0830

Oncogene (1992), 7, 2549–2553

SHORT REPORT

Overexpression of the c-*MET*/HGF receptor gene in human thyroid carcinomas

M.F. Di Renzo[1], M. Olivero[1], S. Ferro[1], M. Prat[1], I. Bongarzone[2], S. Pilotti[3], A. Belfiore[4], A. Costantino[4], R. Vigneri[4], M.A. Pierotti[2] & P.M. Comoglio[1]

[1]*Department of Biomedical Science and Oncology, University of Torino Medical School, Torino, Italy;* [2]*Experimental Oncology A and* [3]*Pathology Departments, National Cancer Institute, Milano, Italy;* [4]*Endocrinology Department, University of Catania Medical School, Catania, Italy*

The receptor for Hepatocyte Growth Factor is a transmembrane tyrosine kinase encoded by the c-*MET* oncogene. We have previously shown that the *Met* protein is expressed in several human epithelial tissues. The receptor is barely detectable, however, in normal thyroids and in specimens from patients affected by non-neoplastic thyroid diseases. Now we report that the expression of the *Met*/HGF receptor is increased a hundred fold in 22 out of 41 human carcinomas derived from the thyroid follicular epithelium. A comprehensive analysis of 15 cases showed that the overexpressing carcinomas belong to histotype variants correlated with negative prognosis and in all but one case there were evidences of locally advanced disease and/or distant metastases. The 11 benign adenomas and the 5 medullary carcinomas tested were negative. Western blot analysis with monoclonal antibodies directed against either the intracellular or the extracellular receptor domains failed to reveal major structural alterations. Southern blot analysis also demonstrated that the c-*MET* gene was not amplified nor rearranged. These data suggest a role for the overexpression of c-*MET* oncogene in the pathogenesis and progression of thyroid tumors derived from the follicular epithelium.

Oncogenes encoding tyrosine kinases are amplified and/or overexpressed in a number of human cancers. The *ERBB-1* gene was found to be overexpressed in squamous carcinomas (Hendler & Ozanne, 1984; Yamamoto *et al.*, 1986; Lu *et al.*, 1988), in renal cell carcinomas (Weidner *et al.*, 1990*b*) and in glioblastomas (Libermann *et al.*, 1985; Wong *et al.*, 1987). The *ERBB-2* gene is overexpressed in carcinomas of the breast (Slamon *et al.*, 1987), ovary (Slamon *et al.*, 1989), stomach (Park *et al.*, 1989), colon (Cohen *et al.*, 1989) and salivary glands (Cohen *et al.*, 1989). Moreover, in breast, ovary and gastric cancers *ERBB-2* gene overexpression has been associated with poor survival (Slamon *et al.*, 1987; Berchuck *et al.*, 1990; Yonemura *et al.*, 1991). In some cases the overexpression of these oncogenes is associated with gene amplification. *FLG* and *BEK* oncogenes, encoding tyrosine kinase receptors for ligands of the Fibroblast Growth Factor family, were found amplified in 10% of breast cancers (Adnane *et al.*, 1991). Activation of the ABL oncogene by translocation has been reported to occur in 95% and 15% of chronic myelogenous and acute lymphoblastic human leukemias respectively (Ben-Neriah *et al.*, 1986; Croce *et al.*, 1987; Hermans *et al.*, 1987). Moreover, in thyroid carcinomas two oncogenes encoding tyrosine kinase receptors, TRK and RET, have also been found to be activated at high frequency by structural alterations, leading to the formation of fusion sequences (Fusco *et al.*, 1987; Bongarzone *et al.*, 1989; Grieco *et al.*, 1990).

The c-*MET* oncogene encodes a transmembrane tyrosine kinase identified as the receptor for a polypeptide known as Hepatocyte Growth Factor (HGF; Bottaro *et al.*, 1991; Naldini *et al.*, 1991*a*) or Scatter Factor (SF; Naldini *et al.*, 1991*b*). HGF/SF is a potent mitogen for epithelial cells (Zarnegar *et al.*, 1989*a*; Miyazawa *et al.*, 1989; Nakamura *et al.*, 1989) and promotes cell motility and invasion (Stoker *et al.*, 1987; Weidner *et al.*, 1990*a*). The *Met*/HGF receptor has a unique structure, being a 190 kDa heterodimer of two disulphide-linked subunits: an extracellular 50 kDa α chain and a transmembrane 145 kDa β chain (Giordano *et al.*, 1989*a*), endowed with tyrosine kinase activity (Gonzatti-Haces *et al.*, 1988). The receptor is synthesized as a 170 kDa precursor that is glycosylated and cleaved to give the mature heterodimer (Giordano *et al.*, 1989*b*).

The c-*MET* oncogene has been originally identified in rearranged form by transfection of DNA from a human osteosarcoma cell line treated *in vitro* with a chemical carcinogen (Cooper *et al.*, 1984; Park *et al.*, 1986). The c-*MET* proto-oncogene was also found to be constitutively activated in a human gastric carcinoma cell line where it is amplified and overexpressed (Giordano *et al.*, 1988; Ponzetto *et al.*, 1991). We have previously shown that the *Met*/HGF receptor is selectively expressed in several normal human epithelial tissues as well as in carcinomas (Di Renzo *et al.*, 1991; Prat *et al.*, 1991*a*). In this work we report that the *Met*/HGF receptor is overexpressed at high frequency in thyroid carcinomas derived from the follicular epithelium.

Results and Discussion

The expression of the Met protein was examined in a series of human tissues obtained from individuals elected as organ donors and immediately frozen. Specimens of normal thyroid obtained from different individuals were compared with samples of other epithelial

Correspondence: M.F. Di Renzo
Received 7 April 1992; accepted in revised form 28 July 1992

tissues (Figure 1). Total protein separated by SDS-PAGE in the presence of reducing agents were analysed by Western blotting. In these conditions the two disulphide-linked chains of the 190 kDa complex are resolved. The β chain of 145 kDa was labelled by an antiserum raised against a synthetic peptide corresponding to the C-terminal tail of the human receptor. The *Met*/HGF receptor was barely detectable in thyroid, while high levels were found in liver, intestine, prostate and uterus (Figure 1). No immunoreactivity in either follicular or parafollicular cells was detected, when thyroids were examined by immunofluorescence microscopy with anti-Met monoclonal antibodies (Prat *et al.*, 1991*a*). Thus the data obtained from Western blotting and from immunohistochemistry experiments are consistent with the presence of an extremely low number of *Met*/HGF receptors homogeneously distributed among the thyroid epithelial cells. Thyroids affected by non-neoplastic diseases, such as simple and multinodular goiters and Graves' disease, were also examined (Figure 1, Table 1). In the latter, as in normal thyroid, the *Met*/HGF receptor was almost undetectable.

The expression of the *Met*/HGF receptor in thyroid tumors was investigated by Western blot analysis of total proteins from surgical specimens, labelled by the antiserum as above. Twenty-two out of the 46 carcinomas studied were scored positive (Table 1). All the medullary (five) and the anaplastic (five) carcinomas examined were negative. Among the 11 adenomas tested, none expressed an elevated level of *Met*/HGF receptor. The overexpression of the *Met*/HGF receptor was in the order of a 100-fold increase (Figure 2). All the positive tumors originated from the follicular epithelium (Table 1).

Fifteen carcinomas were further sub-classified according to their histological and clinical features (Sakamoto *et al.*, 1983; Hermanek & Sobin, 1987; Johnson *et al.*, 1988; Sobrinho-Simoes *et al.*, 1988): all the positives showed evidences of an aggressive phenotype at both histological and clinical levels (Table

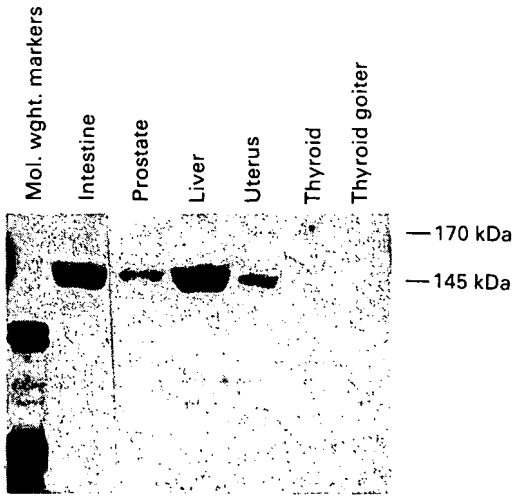

Figure 1 Western blot analysis of the *Met*/HGF receptor expressed by normal human tissues. Tissues were pulverized using a Mikro-Dismembrator (B-Braun) in the presence of liquid nitrogen. Four hundred μg of proteins, solubilized from the whole powdered tissue in boiling Laemmli buffer (Laemmli, 1970) containing the reducing agent β-mercaptoethanol, were loaded onto each lane. Proteins were separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose sheets. Western blot analysis was carried out as described by Towbin *et al.* (1979). Nitrocellulose sheets were labelled with a rabbit polyclonal antiserum raised against a peptide corresponding to nineteen C-terminal amino acids (from Ser^{1372} to Ser^{1390}) of the c-*MET* sequence (EMBL Data-Bank reference n° X54559), followed by ^{125}I-labelled Protein A. X-ray films were exposed for 10 days with intensifying screens. The size of proteins was estimated by using phosphorylase b (92.5 kDa), bovine serum albumin (69 kDa), egg albumin (43 kDa) which had been prelabelled by [^{14}C]methylation as markers (Amersham)

Table 1 Overexpression of the *Met*/HGF receptor in human thyroid tissues

Samples	*No. positive/ No. tested*
Normal thyroids	0/12
Non-neoplastic diseases[a]	0/19
Adenomas	0/11
Medullary carcinoma	0/5
Carcinomas of the follicular epithelium:	
Follicular carcinoma	2/9
Papillary carcinoma	17/23
Poorly differentiated carcinoma	3/4
Anaplastic carcinoma	0/5

[a]These include 10 cases of Graves' disease and 9 multinodular goiters.

Table 2 *MET* overexpression and *TRK* and *RET* rearrangements in thyroid carcinomas derived from the follicular epithelium

Patient	*Sex*	*Age*	*Histology*[a]	*TNM*[b]	*MET*[c]	*RET*[d]	*TRK*[d]	*Follow-up (patient status and duration*[e]*)*
91	F	69	Pap. ca	$T_3N_0M_0$	–	–	–	Alive, no evidence of disease (60)
96	M	18	Pap. ca., columnar var.	$T_4N_{1a}M_1$(PUL)	+	+	–	Alive, with disease (24)
98	F	44	Pap. ca., microcarcinoma var.	$T_1N_0M_0$	–	–	–	Alive, no evidence of disease (48)
110	M	43	Poorly differentiated ca.	$T_3N_0M_1$(OSS, PUL)	+	–	–	Alive, with disease (36)
112	F	23	Pap. ca., tall cell var.	$T_2N_0M_0$	+	–	+	Alive, no evidence of disease (42)
121	M	57	Foll. ca., widely invasive var.	$T_3N_0M_1$(OSS)	–	–	–	Alive, with disease (42)
128	F	43	Pap. ca., follicular var.	$T_4N_{1b}M_0$	+	–	–	Lost
137	M	15	Pap. ca.	$T_4N_{1b}M_1$(OSS)	–	–	–	Lost
157	F	66	Foll. ca., widely invasive var.	$T_4N_0M_1$(OSS)	+	–	–	Lost
162	F	37	Pap. ca., columnar cell var.	$T_4N_{1b}M_0$	+	+	–	Alive, no evidence of disease (24)
167	M	59	Foll. ca., widely invasive var.	$T_2N_0M_0$	–	–	–	Lost
193	F	57	Foll. ca., widely invasive var.	$T_2N_{1a}M_0$	–	–	–	Alive, no evidence of disease (19)
204	F	66	Foll. ca., widely invasive var.	$T_3N_0M_0$(R2)	–	–	–	Alive, with disease (12)
212	M	44	Pap. ca., follicular var.	$T_4N_{1b}M_0$(R2)	–	+	–	Alive, with disease (12)
217	F	74	Poorly differentiated ca.	$T_4N_0M_0$	+	–	–	Alive, no evidence of disease (9)

[a]Abbreviations: Pap. ca., papillary carcinoma; Foll. ca., follicular carcinoma; var., variant. [b]Pathological TNM according to the TNM classification of malignant tumors (Hermanek & Sobin, 1987). [c]Overexpression detected by Western blot analysis. [d]Rearrangement detected by Southern blot analysis. [e]Months.

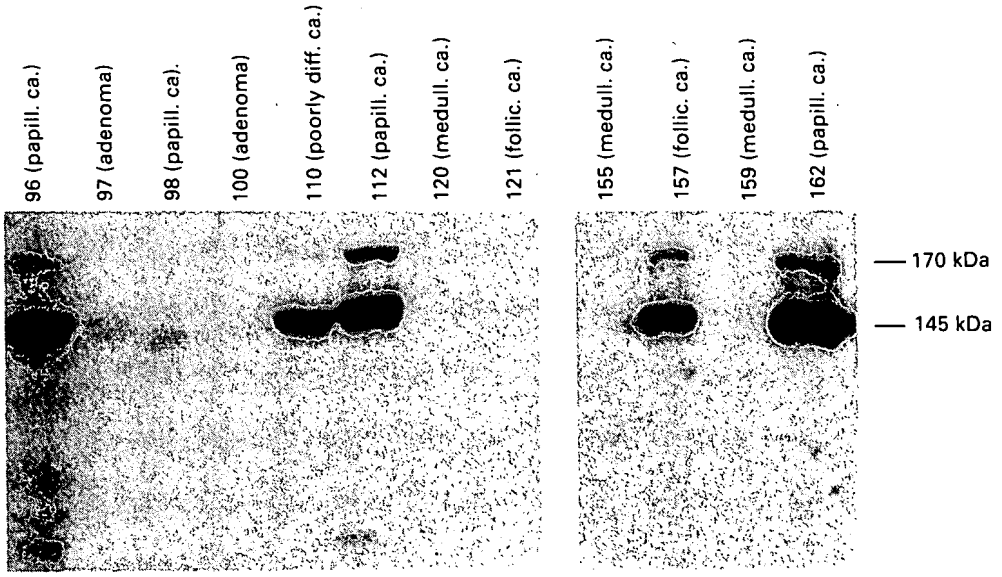

Figure 2 Western blot analysis of the *Met*/HGF receptor expressed by thyroid tumors. Two hundred μg of total proteins, solubilized from samples as described in the legend to Figure 1, were loaded onto each lane. Blots were probed with the antiserum described in the legend to Figure 1. Numbers on the top of each lane indicate different patients. The classification of the tumors analysed is reported in brackets (Papill. ca., papillary carcinoma; Follic. ca., follicular carcinoma; Medull. ca., medullary carcinoma. Poorly diff. ca., poorly differentiated carcinoma). X-Ray films were exposed for 4 days.

2). Three were papillary carcinomas belonging to histological subtypes with aggressive behaviour (i.e. the 'tall cell' variant, Johnson *et al.*, 1988; and the 'columnar cell' variant, Sobrinho-Simoes *et al.*, 1988, now also considered as poorly differentiated papillary carcinoma subtypes). The fourth papillary carcinoma showed evidence of an unusual vascular invasion. Of the other cases, two were classified as poorly differentiated carcinomas (Sakamoto *et al.*, 1983) and one as a widely invasive follicular carcinoma (Hediger, 1988). With regard to the extent of the disease (Table 2), three had distant metastases at presentation (M1) and four showed evidence of extracapsular infiltration (T4).

To ascertain whether the *Met*/HGF receptor overexpressed in these thyroid tumors had structural features of the authentic receptor, proteins were labelled with two different monoclonal antibodies. The DQ-13 antibody was directed against the C-terminal tail; the DL-21 bound an epitope located in the extracellular domain (Prat *et al.*, 1991*b*). Both the epitopes were present in the *Met* receptor expressed by the tumors, showing that the protein did not undergo major structural alterations. The DL-21 antibody also detected the 85 kDa β chain variant lacking the cytoplasmic domain (Figure 3). This truncated form has been previously described: its physiological role in modulating *Met*/HGF receptor functions has been discussed elsewhere (Prat *et al.*, 1991*b*).

To investigate whether the overexpression of the *Met*/HGF receptor, observed in the thyroid tumors, was due to c-*MET* gene rearrangement or amplification, Southern blot analysis was performed using a cDNA encompassing the entire c-*MET* coding sequence as a probe. Identical restriction patterns were detected in the tumors after EcoRI or HindIII digestion (data not shown). The same fragments were obtained by digestion of DNA from a reference cell line where the *MET* cDNA was cloned and sequenced (Ponzetto *et al.*, 1991). No evidence for amplification was found judging from the intensity of the DNA bands obtained from the tumors overexpressing the *Met*/HGF receptor.

The molecular basis of the carcinogenesis of thyroid follicular epithelium is largely unknown. The occurrence of RAS mutations in both benign adenomas and

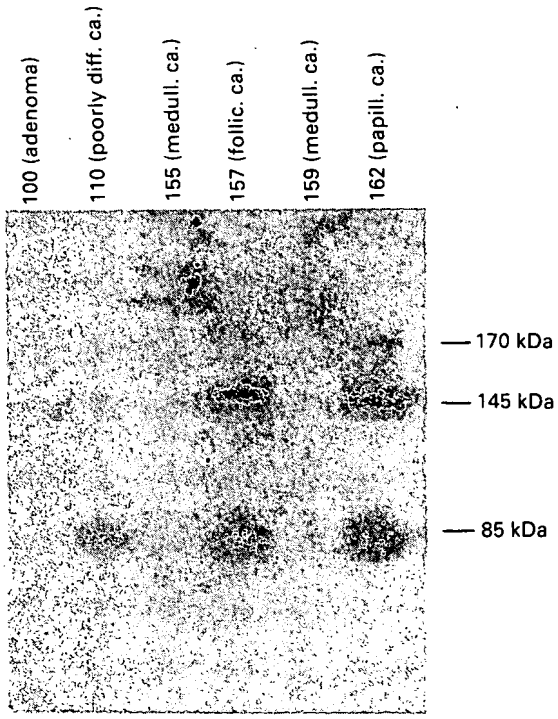

Figure 3 Western blot analysis of the *Met*/HGF receptor expressed by thyroid tumors. Two hundred μg of total proteins solubilized from samples were loaded onto each lane. Blots were probed with monoclonal antibodies directed against the extracellular domain of the *Met* β chain (Prat *et al.*, 1991*b*), followed by ^{125}I-labelled Rabbit anti-mouse Ig immunoglobulins. X-ray films were exposed for 6 days with intensifying screens. The samples are classified as in Figure 2.

carcinomas strengthens the hypothesis that this oncogene is involved in the early steps (Lemoine *et al.*, 1988, 1989, 1990; Suarez *et al.*, 1988, 1990). Recently, the mutation of the gene encoding the stimulatory subunit of adenyl-cyclase (*GSP*) has been added to the list of the molecular events contributing to the malignant phenotype (Suarez *et al.*, 1991). The TRK and RET oncogenes, both encoding tyrosine kinases, were found rearranged in 50% of the papillary carcinomas; the rearrangements of these genes correlate with their transforming activity detectable in transfection assay (Fusco *et al.*, 1987; Bongarzone *et al.*, 1989; Grieco *et al.*, 1990; Greco *et al.*, 1992). Therefore 15 out of the 46 carcinomas examined in this work were also investigated for *TRK* and *RET* rearrangements, using the previously described probe-restriction enzyme combination (Bongarzone *et al.*, 1989). Interestingly, among the papillary tumors overexpressing the c-*MET* oncogene, one had also rearranged TRK and two had rearranged RET sequences. RET was also rearranged in one of the papillary carcinomas negative for c-*MET* overexpression (Table 2). This gave strength to the possible involvement of tyrosine kinase oncogenes in the papillary type of thyroid carcinomas. In our series more than 70% of the papillary carcinomas showed overexpression of the *MET* gene and 2 out of the three poorly differentiated carcinomas showed areas of papillary differentiation. Both follicular and papillary carcinomas are believed to originate from the same follicular cells of the thyroid epithelium though they have different pathogenic features and clinical behaviour. The follicular carcinoma is associated with endemic goiter, low iodine intake and metastasizes almost exclusively via the blood-stream, whereas the papillary carcinoma is associated with high iodine intake and metastasizes by lympathic spread. Altogether, these data suggest that activated tyrosine kinases may have a role in determining the different properties of the tumors derived from the thyroid follicular epithelium.

The c-*MET* gene is a receptor-type tyrosine kinase oncogene, originally identified as a transforming gene activated by rearrangement in cells treated with a chemical carcinogen (Cooper *et al.*, 1984; Dean *et al.*, 1985). c-*MET* was also found to be activated by amplification and overexpression (Giordano *et al.*, 1989*a*; Ponzetto *et al.*, 1991), suggesting that an inappropriate number of receptors at the cell surface might trigger its transforming potential. It seems thus reasonable to propose that overexpression of the *Met*/HGF receptor by neoplastic thyroid follicular cells might sustain their growth given the presence of HGF. In these tumors, a paracrine relationship could be postulated, since Zarnegar *et al.* (1989*b*) have shown that thyroid parafollicular-C cells secrete HGF. Since the c-*MET* oncogene is overexpressed at high frequency in papillary and in poorly differentiated carcinomas, but not in adenomas, it is suggested that this gene, originally described following activation *in vitro*, can play a role in the onset or, more likely, in the progression of human cancers of specific histotypes. Recently, HGF has been identified with Scatter factor (Naldini *et al.*, 1991*b*; Weidner *et al.*, 1991), a polypeptide which mediates cell motility and invasion in a number of epithelial as well as carcinoma cells (Stoker *et al.*, 1987; Weidner *et al.*, 1990*a*). Overexpression of the *Met*/HGF receptor may confer to thyroid carcinomas the ability to progress towards malignancy through the acquisition of a more aggressive behaviour.

Acknowledgements

We are indebted to Professor G. Della Porta for helpful suggestion and criticism. We thank Dr C. Ponzetto for critical reading of the manuscript. The excellent technical assistance of L. Palmas and R. Callipo are gratefully acknowledged. This work was supported by the Italian Association for Cancer Research (A.I.R.C.) and by the Italian National Research Council (C.N.R., Progetto Finalizzato A.C.R.O.).

References

Adnane, J., Gaudray, P., Dionne, C.A., Crumley, G., Jaye, M., Schlessinger, J., Jeanteur, P., Birnbaum, D. & Theillet, C. (1991). *Oncogene*, **6**, 659–663.

Ben-Neriah, Y., Daley, G.Q., Mes-Masson, A.M., Witte, O.N. & Baltimore, D. (1986). *Science*, **233**, 212–215.

Berchuck, A., Kamel A., Whitaker, R., Kerns, B., Olt, G., Kinney, R., Soper, J.T., Dodge, R., Clarke-Pearson, D.L., Marks, P., McKenzie, S., Yin, S. & Bast, R.C. Jr. (1990). *Cancer Res.*, **50**, 4087–4091.

Bongarzone, I., Pierotti, M.A., Monzini, N., Mondellini, P., Manenti, G., Donghi, R., Pilotti, S., Grieco, M., Santoro, M., Fusco, A., Vecchio, G. & Della Porta, G. (1989). *Oncogene*, **4**, 1457–1462.

Bottaro, D.P., Rubin, J.S., Faletto, D.L., Chan, A.M.L., Kmiecik, T.E., Vande Woude, G.F. & Aaronson, S.A. (1991). *Science*, **251**, 802–804.

Cohen, J.A., Weiner, D.B., More, K.F., Kokai, Y., Williams, W.v., Maguire, H.C., LiVolsi, V. & Greene, M. (1989). *Oncogene*, **4**, 81–88.

Cooper, C.S., Park, M., Blair, D., Tainsky, M.A., Huebner, K., Croce, C.M. & Vande Woude, G.F. (1984). *Nature*, **311**, 29–33.

Croce, C.M., Huebner, K., Isolbe, M., Fainstain, E., Lifshitz, B., Shtivelman, E. & Canaani, E. (1987). *Proc. Natl. Acad. Scia USA*, **84**, 7174–7179.

Dean, M., Park, M., Le Beau, M.M., Robins, T.S., Diaz, M.O., Rowley, J.D., Blair, D.G. & Vande Woude, G.F. (1985). *Nature*, **318**, 385–388.

Di Renzo, M.F., Narsimham, R.P., Olivero, M., Bretti, S., Giordano, S., Medico, E., Gaglia, P., Zara, P. & Comoglio, P.M. (1991). *Oncogene*, **6**, 1997–2003.

Fusco, A., Grieco, M., Santoro, M., Berlingeri, M.T., Pilotti, S., Pierotti, M.A., Della Porta, G. & Vecchio, G.C. (1987). *Nature*, **328**, 170–172.

Giordano, S., Di Renzo, M.F., Ferracini, R., Chiado'Piat, L. & Comoglio, P.M. (1988). *Mol. Cell. Biol.*, **8**, 3510–3517.

Giordano, S., Ponzetto, C., Di Renzo, M.F., Cooper, C.S. & Comoglio, P.M. (1989*a*). *Nature*, **339**, 155–156.

Giordano, S., Di Renzo, M.F., Narsimham, R., Cooper, C.S., Rosa, C. & Comoglio, P.M. (1989*b*).*Oncogene*, **4**, 1383–1388.

Gonzatti-Haces, M., Seth, A., Park, M., Copeland, T., Oroszlan, S. & Vande Woude, G.F. (1988). *Proc. Natl. Acad. Sci. USA*, **85**, 21–25.

Greco, A., Pierotti, M.A., Bongarzone, I., Pagliardini, S., Lanzi, C. & Della Porta, G. (1992). *Oncogene*, **7**, 237–242.

Grieco, M., Santoro, M., Berlingeri, M.T., Melillo, R.M., Donghi, R., Bongarzone, I., Pierotti, M.A., Della Porta, G., Fusco, A. & Vecchio, G. (1990). *Cell*, **60**, 557–563.

Hedigar, C., Williams, E.D. & Sobin, L.H. (1988). *Histological Typing of Thyroid Tumors*. Springer-Verlag:Berlin Heidelberg.

Hendler, F.J. & Ozanne, B.W. (1984). *J. Clin. Invest.*, **74**, 647–651.

Hermanek, P. & Sobin, L.H. (1987). *TNM Classification of Malignant Tumor*, U.I.C.C.:Geneva.

Hermans, A., Heisterkamp, N., von Lindern, M., van Baal, S., Meijer, D., van der Plas, D., Wiedemann, L.M., Groffen, J., Bootsma, D. & Grosveld, G. (1987). *Cell*, **51**, 33–40.

Johnson, T.L., Lloyd, R.V., Thompson, N.W., Beierwaltes, W.H. & Sisson, J.C. (1988). *Am. J. Surg. Pathol.*, **12**, 22–27.

Laemmli, U.K. (1970). *Nature*, **230**, 680–685.

Libermann, T.A., Nusbaum, H.R., Razon, N., Kris, R., Lax, I., Soreq, H., Whittle, N., Waterfield, M.D., Ullrich, A. & Schlessinger, J. (1985). *Nature*, **313**, 144–147.

Lemoine, N.R., Staddon, S., Bond, J., Wyllie, F.S., Shaw, J.J. & Wynford-Thomas, D. (1990). *Oncogene*, **5**, 1833–1837.

Lemoine, N.R., Mayall, E.S., Wyllie, F.S., Farr, C.J., Hughes, D., Padua, R.A., Thurston, V., Williams, E.D. & Wynford-Thomas, D. (1988). *Cancer Res.*, **48**, 4459–4463.

Lemoine, N.R., Mayall, E.S., Wyllie, F.S., Williams, E.D., Goyns, M., Stringer, B. & Wynford-Thomas, D. (1989). *Oncogene*, **4**, 159–164.

Lu, S.-H., Hsieh, L.L., Luo, F.C. & Wienstein, I.B. (1988). *Int. J. Cancer*, **42**, 502–505.

Miyazawa, K., Tsuboochi, H., Naka, D., Takahashi, K., Okigaki, M., Arikaki, N., Nakayama, H., Hirono, S., Sakiyama, O., Gohoda, E., Daikuhara, Y. & Kitamura, N. (1989). *Biochem. Biophys. Res. Commun.*, **163**, 967–973.

Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K. & Shimizu, S. (1989). *Nature*, **342**, 442–443.

Naldini, L., Vigna, E., Narsimhan, R., Gaudino, G., Zarnegar, R., Michalopoulos, G.K. & Comoglio, P.M. (1991*a*). *Oncogene*, **6**, 501–504.

Naldini, L., Weidner, K.M., Vigna, E., Gaudino, G., Bardelli, A., Ponzetto, C., Narsimhan, R.P., Hartmann, G., Zarnegar, R., Michalopoulos, G.K., Birchmeier, W. & Comoglio, P.M. (1991*b*). *EMBO J.*, **10**, 2867–2878.

Park, M., Dean, M., Cooper, C.S., Schmidt, M., O'Brian, S.J., Blair, D.G. & Vande Woude, G.F. (1986). *Cell*, **45**, 895–904.

Park, J.B., Rhim, J.S., Park, S.C., Kimm, S.W. & Kraus, M.H. (1989). *Cancer Res.*, **49**, 6605–6609.

Ponzetto, C., Giordano, S., Peverali, F., Della Valle, G., Abate, M., Vaula, G. & Comoglio, P.M. (1991). *Oncogene*, **6**, 553–559.

Prat, M., Narismhan, R.P., Crepaldi, T., Nicotra, M.R., Natali, P.G. & Comoglio, P.M. (1991*a*). *Int. J. Cancer*, **49**, 323–328.

Prat, M., Crepaldi, T., Gandino, L., Giordano, S., Longati, P. & Comoglio, P.M. (1991*b*). *Mol. Cell. Biol.*, **11**, 5954–5962.

Sakamoto, A.S., Kasai, N. & Sugano, H. (1983). *Cancer*, **52**, 1849–1855.

Slamon, D.J., Clark, J.M., Wong, S.C., Levin, W.J., Ullrich, A. & McGuire, L. (1987). *Science*, **235**, 177–182.

Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.C., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A. & Press, F.M. (1989). *Science*, **244**, 707–712.

Sobrinho-Simoes, M., Nesland, J.M. & Johannessen, J.V. (1988). *Clin. Pathol.*, **89**, 264–267.

Stoker, M., Gherardi, E., Perryman, M. & Gray, J. (1987). *Nature*, **327**, 239–242.

Suarez, H.G., du Villard, J.A., Caillou, B., Schlumbemberger, M., Tubiana, M., Parmentier, C. & Monier, R. (1988). *Oncogene*, **2**, 403–406.

Suarez, H.G., du Villard, J.A., Severino, M., Caillou, B., Schlumbenberger, M., Tubiana, M., Parmentier, C. & Monier, R. (1990). *Oncogene*, **5**, 565–570.

Suarez, H.G., du Villard, J.A. Caillou, B., Schlumbemberger, M., Parmentier, C. & Monier, R. (1991). *Oncogene*, **6**, 677–679.

Towbin, H., Staehelin, T. & Gordon, J. (1979). *Proc. Natl Acad. Sci. USA*, **76**, 4350–4353.

Weidner, K.M., Behrens, J., Vanderkerckhone, J. & Birchmaier, W. (1990a). *J. Cell Biol.*, **111**, 2907–2911.

Weidner, U., Peter, S., Strohmeyer, T., Hussnatter, R., Ackermann, R. & Sies, H. (1990*b*). *Cancer Res.*, **50**, 4504–4509.

Weidner, K.M., Arakaki, N., Hartmann, G., Vanderkerckhone, J., Weingert, S., Rieder, H., Fonatsch, C., Tsubouchi, H., Hishida, T., Daikuhara, Y. & Birchmaier, W. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 7001–7005.

Wong, A.J., Bigner, S.H., Bigner, D., Kinzler, K.W., Hamilton, S.R. & Vogelstein, B. (1987). *Proc. Natl. Acad. Sci. USA*, **84**, 6899–6903.

Yamamoto, T., Kamata, N., Kawano, H., Shimizu, S., Kuroki, T., Toyoshima, K., Rikimaru, K., Nomura, N., Ishizaki, R., Pastan, I., Gamou, S. & Shimizu, N. (1986). *Cancer Res.*, **46**, 414–416.

Yonemura, Y., Ninomiya, I., Yamaguchi, A., Fushida, S., Kimura, H., Ohoyama, S., Miyazaki, I., Endou, Y., Tanaka, M. & Sasaki, T. (1991). *Cancer Res.*, **51**, 1034–1038.

Zarnegar, R., Muga, S., Enghild, J. & Michalopoulos, G. (1989*a*). *Biochem. Biophys. Res. Commun.*, **163**, 1370–1376.

Zarnegar, R., Muga, S., Rahija, R.J. & Michalopoulos, G. (1989*b*). *Proc. Natl. Acad. Sci. USA*, **87**, 1252–1256.

NPL___ ADONIS___ MIC___ BioT___Main__NO X Vol NO__ NOS___CKCite__Dup__Int SAS

4-2-0

STIC-ILL

From: Davis, Minh-Tam
Sent: Monday, April 02, 2007 4:06 PM
To: STIC-ILL
Subject: Reprint request for 10/500297

623570

1) Presence and tyrosine phosphorylation of c-met receptor in human sperm.
Herness E A; Naz R K
Department of Obstetrics and Gynecology, Medical College of Ohio, Toledo 43614-5806, USA.
Journal of andrology (UNITED STATES) Sep-Oct 1999, 20 (5) p640-7, ISSN 0196-3635--Print Journal Code: 8106453
Contract/Grant No.: HD24425; HD; NICHD

COMPLETED

2) HGF induces FAK activation and integrin-mediated adhesion in MTLn3 breast carcinoma cells.
Beviglia L; Kramer R H
Department of Stomatology, University of California, San Francisco, San Francisco, CA, USA.
International journal of cancer. Journal international du cancer (UNITED STATES) Nov 26 1999, 83 (5) p640-9, ISSN 0020-7136--Print Journal Code: 0042124

2224526,

3) Title: OVEREXPRESSION OF THE C-MET HGF RECEPTOR GENE IN HUMAN THYROID CARCINOMAS (Abstract Available)
Author(s): DIRENZO MF; OLIVERO M; FERRO S; PRAT M; BONGARZONE I; PILOTTI S; BELFIORE A; COSTANTINO A; VIGNERI R; PIEROTTI MA; COMOGLIO PM
Corporate Source: UNIV TURIN,SCH MED,DEPT BIOMED SCI & ONCOL/I-10124 TURIN//ITALY/; NATL CANC INST,DEPT EXPTL ONCOL A/MILAN//ITALY/; NATL CANC INST,DEPT PATHOL/MILAN//ITALY/; UNIV CATANIA,SCH MED,DEPT ENDOCRINOL/I-95124 CATANIA//ITALY/
Journal: ONCOGENE, 1992, V7, N12 (DEC), P2549-2553
ISSN: 0950-9232

4) 268602 PMID: 9065649
Detection of MET oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell carcinomas.
Galeazzi E; Olivero M; Gervasio F C; De Stefani A; Valente G; Comoglio P M; Di Renzo M F; Cortesina G
Department of Clinical Physiopathology, University of Turin School of Medicine, Italy.
European archives of oto-rhino-laryngology - official journal of the European Federation of Oto-Rhino-Laryngological Societies (EUFOS) - affiliated with the German Society for Oto-Rhino-Laryngology - Head and Neck Surgery (GERMANY) 1997, 254 Suppl 1 pS138-43, ISSN 0937-4477--Print Journal Code: 9002937

THANK YOU.

MINH TAM DAVIS
ART UNIT 1642, REMSEN 3A24, MB 3C18
272-0830

Eur Arch Otorhinolaryngol (1997) 254 (Suppl. 1): S138–S143

ONCOLOGY

E. Galeazzi · M. Olivero · F. C. Gervasio
A. De Stefani · G. Valente · P. M. Comoglio
M. F. Di Renzo · G. Cortesina

Detection of *MET* oncogene/hepatocyte growth factor receptor in lymph node metastases from head and neck squamous cell carcinomas

Abstract The c-*MET* oncogene encodes the receptor for hepatocyte growth factor/scatter factor (HGF/SF), which is known to stimulate the invasive growth of epithelial cells cultured in vitro. The *Met*/HGF receptor is a heterodimeric transmembrane tyrosine kinase, which is a prototype for a new family of growth factor receptors. The c-*MET* oncogene is expressed in several types of epithelial tissue including keratinocytes and is over-expressed in a number of human carcinomas. Studies on various carcinoma cell lines have shown that over-expression and structural alteration of the receptor result in its activation and confer tumorigenesis. We have studied *Met*/HGF receptor expression in tissue specimens from 34 patients with head and neck squamous cell carcinomas (HNSCC) and in 17 regional lymph node metastases. Western blot analysis was employed, using monoclonal antibodies directed against either the intracellular or extracellular domain of the receptor. Each sample was compared to its normal counterpart. The receptor did not show any major structural alterations in HNSCC tissues, but its expression was increased from 2- to 50-fold in about 70% of tumors. Immunohistochemistry then showed that the same antibodies stained only a few cells in the basal layer of normal squamous epithelium but intensely marked tumor cells. In the lymph node metastases of Met-positive tumors, receptor expression was maintained and sometimes increased with respect to primary tumors. Immunohistochemical analysis of the metastatic lymph nodes showed that cells were negative in the normal lymphatic tissue and strongly stained in tumor cells. Over-expression of the *Met*/HGF receptor was found at all tumor stages but was more significant in those associated with enlarged or multiple (N2–N3) lymph node metastases. These data show that expression of the *Met*/HGF receptor may be involved in the progression of HNSCC towards a metastatic phenotype and may be a useful marker of head and neck tumor cell spread to regional lymph nodes.

Key words Lymph node metastases · *MET* oncogene · Head and neck squamous cell carcinoma · Hepatocyte growth factor receptor

E. Galeazzi · F. C. Gervasio · A. De Stefani · G. Cortesina
Department of Clinical Physiopathology,
University of Turin School of Medicine, Turin, Italy

M. Olivero · G. Valente · P. M. Comoglio
Department of Biomedical Sciences and Oncology,
University of Turin School of Medicine, Turin, Italy

M. F. Di Renzo
Institute of Histology, University of Sassari School of Medicine,
Sassari, Italy

G. Cortesina (✉)
Clinica ORL, Via Genova 3, I-10126 Turin, Italy

Introduction

The c-MET oncogene [6] encodes a transmembrane tyrosine kinase, which has now been identified as the receptor for hepatocyte growth factor (HGF) or "scatter factor" (SF) [14]. This receptor (*Met*/HGF receptor) is an $\alpha\beta$ complex of 190 kDa and is composed of an extracellular 50 kDa α-chain and a 145 kDa β-chain [13] that spans the plasma membrane and shows tyrosine kinase activity [20]. These two chains are disulfide-linked. The ligand of the receptor encoded by the *MET* gene, HGF/SF, has pleiotropic effects on epithelial cells. It is mitogenic and also stimulates cell motility, dissociation of epithelial sheets and invasion of the cellular matrix [26].

The *Met*/HGF receptor plays a significant role in cell physiology during development and tissue regeneration and is expressed in several normal human epithelial tissues, including those of the liver, kidney and lung [9, 22]. Activation of the *Met*/HGF receptor kinase leads to uncontrolled proliferation of cells. The over-expression of the *Met* protein is most probably sufficient for the constitutive activation of *Met* kinase with the *MET* oncogene activated by amplification or structural alterations of the extracellular domain of the receptor.

Activation of the c-*MET* oncogene has been related to tumor cell invasiveness and metastasis on the basis of ex-

periments carried out in animal models and cells cultured in vitro. We have shown that the c-*MET* gene is often over-expressed in human carcinomas [10, 11]. In colorectal carcinoma, for example, the expression of the *MET* gene was increased from 5- to 50-fold in about 50% of tumors and in 70% of liver metastases. While over-expression was associated with amplification in only 10% of primary tumors, 8 of 9 liver metastases showed amplification (2- to 3-fold) of the *MET* gene [11]. These data suggest that the *MET* oncogene and its ligand HGF/SF may be involved in the onset, and, more probably, progression of human cancers of epithelial origin to confer a selective growth advantage to neoplastic cells.

Cancers of the head and neck represent a number of diseases that share similar biological, pathological and clinical features [24]. The great majority of these tumors derive from squamous epithelium and disseminate primarily to regional lymph nodes. Since disease evolves through metastatic spread in lymph nodes, a correct assessment of its extent and lymph node involvement is of great importance for the effective clinical management of patients. In so doing, the use of molecular markers to detect lymph node metastases may help in diagnosis and planning therapy. Oncogene abnormalities are the most useful cancer markers, since they are directly correlated to the presence of transformed cells and may help in identifying a low number of transformed cells in a normal context. In this study, we examined a series of tissue samples taken from 34 patients with head and neck squamous cell carcinomas (HNSCC) and 17 lymph node metastases for the expression of the *Met*/HGF receptor.

Materials and methods

Tissue specimens

Tumor and normal tissues were obtained from the University of Turin pathologist at the time of surgery on patients (Table 1). Each specimen was treated according to its subsequent use. Samples were snap-frozen and pulverized in the presence of liquid nitrogen for Western blot analysis, ornithine carbamyltransferase-compound-embedded (O.C.T. compound, Miles Diagnostics Division, Elkhart, Ind., USA) and immediately frozen for immunohistochemistry. Tissues were paraffin-embedded for histological examinations.

Table 1 Tissue/organ sites examined for tumor *Met*/HGF receptor expression

Site	Number (*n*)	Lymph node metastases
Oral cavity	7	3
Oropharynx	6	4
Hypopharynx	6	4
Larynx	15	3
Cervical esophagus[a]	1	1
Not defined		2

[a] Tumor in same patient with hypopharyngeal cancer

Antibodies

DQ13 anti-*Met*/HGF receptor monoclonal antibodies (mAbs) were used for Western blot analysis and were raised against a peptide corresponding to 19 C-terminal amino acids (from Ser^{1372} to Se^{1390}) of the human receptor [European Molecular Biology Laboratory (EMBL) Data Bank reference no. X54559]. DL21 mAbs (used for Western blot analysis) and DO-24 (used for immunohistochemistry) were both directed against the extracellular domain of the *Met*/HGF receptor. Both DQ13 and DL21 antibodies for molecular analysis were employed to confirm that the receptor did not undergo major structural alterations. All antibodies were kindly provided by Dr. M. Prat and were raised as described elsewhere [21].

Western blotting

Tissues were pulverized in the presence of liquid nitrogen in a B-Braun Mikro Dismembrator and then solubilized in boiling sodium dodecyl sulfate-containing buffer with β-mercaptoethanol as reducing agent. Under these conditions, the 50 kDa α- and the 145 kDa β-chain, which constitute the 190 kDa receptor, were distinct. Equal amounts of proteins (300 μg) were loaded into each lane. Proteins were separated in polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. Western blot analysis was carried out as previously described [9]. Blots were probed with anti-*Met*/HGF receptor protein antibodies and then with horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins, and revealed by enhanced chemiluminescence (ECL, Amersham). The relative expression of the receptor was quantified by laser densitometric scanning of X-ray films. Pathological tissues were always compared to normal mucosa and unaffected lymph nodes of the same patient as a control.

Immunohistochemistry

Fresh tissue samples were snap-frozen in the presence of cryopreservant. Five-micrometer cryostat sections were fixed in cold absolute acetone for 10 min. All stains were performed with a mixture of DO24, DQ13 and DL21 mAbs as hybridoma supernatants. Control samples were incubated with an unrelated mAb of the same isotype. Selected specimens were stained with immunoperoxidase using the ABC Dako Lsab 2 kit according to the manufacturer's instructions. The enzymatic activity was developed using diaminobenzidine as chromogenic substrate for 10 min. Slides were then rinsed with phosphate-buffered saline and counterstained with Mayer's hematoxylin.

Results

Table I lists the main clinico-pathological features of patients and tumors: an increase in expression of the c-*MET* gene was detected in more than 75% of the carcinomas examined. The level of over-expression ranged from 2 to 50 times compared to normal tissues (Fig. 1). A single sample of mucosa containing high-grade dysplasia and one case of adenocarcinoma of the nasal fossa showed no expression of the receptor, while a peristomal recurrence showed an increased level of *Met*/HGF receptor expression with respect to the primary lesion in both Western blot and histopathological analyses (data not shown). The *Met*/HGF receptor was detectable at a very low level in a few of the normal tissue samples.

Table 2 and Fig. 1 show that the *Met*/HGF receptor was consistently detectable in metastatic lymph nodes, whereas

Table 2 Clinico-pathological features of squamous cell carcinomas of the head and neck over-expressing the *Met*/HGF receptor using Western blot analysis for HGF-R

Patient	Age/sex	Site	Stage (pTNM)		HGF-R[a]	Follow-up[b]
GA	57/M	Larynx	rpT4pN0[c]	G2	+	NED
GB	68/M	Oropharynx	pT4pN2b	G2	+	NED
		Synchr. lymph node metastasis			+	
PE	48/M	Hypopharnynx and esophagus	pT4pN2c	G3	+	DOD
		Synchr. lymph node metastasis			+	
GR	58/M	Oral cavity[d]	pT2pN1	G2	+	Not available
DMS	90/M	Larynx	pT4Nx[e]	G2	–	LWD
AA	74/M	Hypopharynx	pT4pN0	G2	–	NED
BM	75/M	Larynx	pT2pN0	G2	+	NED
BG	67/M	Hypopharynx	pT4pN3	G3	++	DOD
		Synchr. lymph node metastasis[1]			+	
		Synchr. lymph node metastasis[2]			+	
VC	67/M	Hypopharynx	pT3pN0	G3	+++	NED
BG	67/M	Larynx	pT3pN0	G3	–	DOD
MG	72/M	Oral cavity	pT4pN0	G3[f]	+++	Not available
GL	72/M	Larynx	pT3pN0	G2	+	NED
AG	53/M	Larynx	pT2pN0	G2	–	NED
MG	75/M	Larynx	pT1bpN0	G1	+	NED
GM	58/M	Metachr. lymph node metastasis[g]	TxpN1	G3	+	NED
RC	71/F	Oral cavity[h]	pT2pN1[i]	G2	+	LWD
		Synchr. lymph node metastasis			+	
CD	51/M	Larynx	pT2pN0	G1	++	NED
SE	60/M	Larynx	pT3pN2b	G2	+	DOD
		Synchr. lymph node metastasis			++	
		Peristomal recurrence			++	
TG	65/M	Metachr. lymph node metastasis	pTN1[j]		++	NED
DMP	60/M	Oropharynx	pT2pN2b[i]	G3	+	LWD
		Synchr. lymph node metastasis			++	
PB	67/M	Oropharynx	pT4pN2b	G2	+	NED
		Synchr. lymph node metastasis			++	
BG	71/M	Oropharynx	pT1pN0	G2	–	NED
AE	72/M	Oral cavity	pT1pN0	G2	+	NED
MG	66/M	Oropharynx	pT2pN2c	G3	+	Not available
		Synchr. lymph node metastasis			++	
MS	67/M	Larynx	pT3pN0	G2	–	NED
CG	57/M	Oral cavity	pT2pN2b	G3	+	NED
		Synchr. lymph node metastasis			++	
PM	43/M	Larynx	pT4pN2c	G2	nd	NED
		Synchr. lymph node metastasis			++	
BI	61/F	Oral cavity	pT4pN0	G1	+	NED
GG	61/M	Hypopharynx	pT4pN1	G3	++	NED
		Synchr. lymph node metastasis			++	
CA	39/M	Oropharynx	pT4pN0[k]	G2	++	LWD
GV	57/M	Larynx	pT3pN2b	G1	+	NED
TC	65/M	Larynx	pT1aNx	G2	++	NED
CL	63/M	Larynx	pT4pN0	G3	++	NED
PE	63/M	Larynx	pT4pN1	G3	–	NED
		Synchr. lymph node metastasis			+++	
CGB	63/M	Oral cavity	pT2pN1	G3	–	NED
		Synchr. lymph node metastasis			++	
FP	53/M	Hypopharynx	pT3pN1	G2	+	NED
		Synchr. lymph node metastasis			+++	

[a] Met/HGF receptor expression scores were: (+) = 2- to 5-fold increase, (++) = 5- to 10-fold increase, (+++) = >10-fold increase

[b] *NED* no evidence of disease, *DOD* dead of disease, *LWD* living with disease

[c] Tumor recurrence after fronto-lateral laryngectomy in 1993 for a pT1pN0 cancer

[d] Patient underwent hemilaryngectomy in 1982 and total laryngectomy in 1985; interleukin-2 treatment was given before surgery for the current tumor (5000 IU per 10 injections)

[e] Lymph node metastasis was detected 6 months after surgery and patient was then treated with radiotherapy

[f] Surgery included laryngectomy plus radiotherapy in 1989

[g] Lymph node metastasis was from an unknown primary tumor

[h] Tumor was carcinoma in a mixed malignant tumor in a minor salivary gland

[i] Lung metastases after surgery

[j] Cervical adenopathy; hemilaryngectomy performed in 1984

[k] Chemotherapy was given before surgery, using CDDP + 5-FU

it was never seen in the unaffected ones. The level of expression of the receptor in the metastatic nodes was similar to and sometimes higher than that found in the corresponding primary tumor. Three enlarged nodes suspected to be metastatic were negative on molecular analysis. Histopathological examination confirmed this diagnosis.

Immunohistochemistry showed that the *Met*/HGF receptor in normal epithelium was localized as weak positive staining in the basal layer. Nine of 12 primary tumors examined showed an increased homogeneous staining that was evident as both membrane and cytoplasmic staining of neoplastic cells. The stroma was stained negatively in

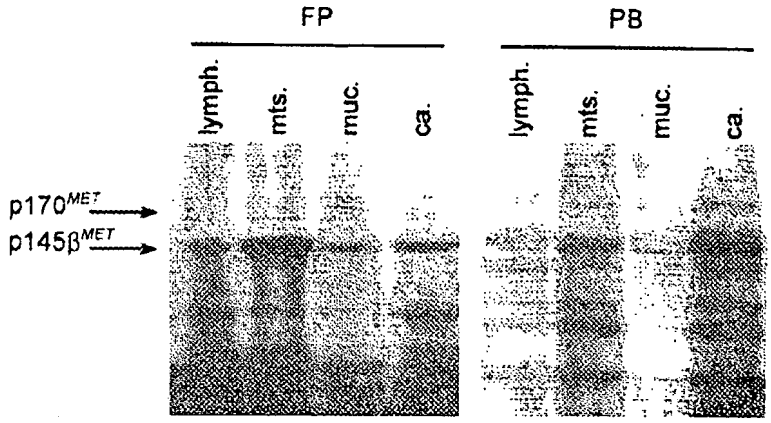

Fig. 1 Western blot analysis of samples of primary squamous cell carcinomas of the head and neck (*ca.*) and metastases (*mts.*) compared to samples of normal mucosa (*muc.*) and unaffected lymph nodes (*lymph*). Initials at the top of the lanes indicate different patients. The DQ-13 antibody, recognizing the p145 β chain and the p170 precursor, was directed against the C-terminal tail of the *Met*/HGF receptor

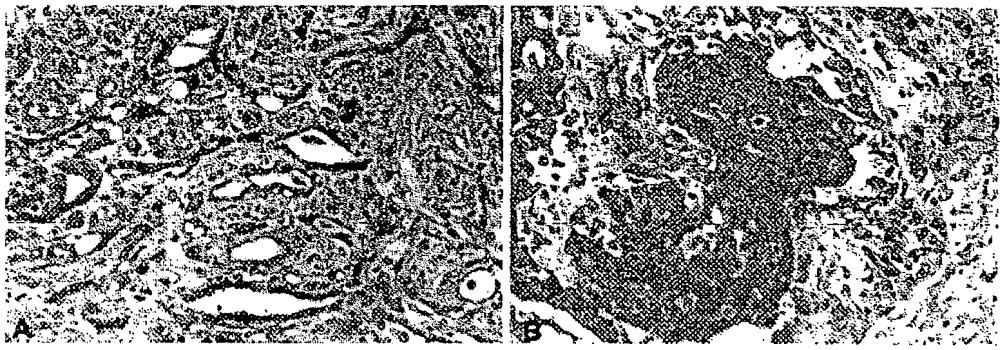

Fig. 2 A, B Pattern of *Met*/HGF receptor immunostaining in primary HNSCC. ABCimmunoenzymatic method. **A** Neoplastic cells arranged in thin cords and a pseudoglandular pattern. The surrounding stroma is negative (× 63). **B** Strong positivity displayed by neoplastic cells arranged in sheets (× 63)

all cases, whereas a weak positive staining was displayed in vessels as the (probable) result of endothelial cell positivity [4]. The intensity of the reaction showed case differences, but was consistent with the quantitative data obtained with Western blot analysis. In primary carcinomas, *Met*-positive neoplastic cells formed pseudoglandular structures (Fig. 2A) or sheets or nests (Fig. 2B). In the 12 metastatic lymph nodes examined, neoplastic cells reproduced the histopathological features and receptor positivity levels of the corresponding primary tumor, although staining was frequently more intense in nodes. By contrast, immunohistochemistry showed that unaffected areas of lymph nodes were completely negative.

T and N staging was also related to the level of *Met*/HGF receptor expression. Clinically, *Met*/HGF receptor over-expression was associated with disease at any stage, as classified according to American Joint Committee on Cancer (AJCC) criteria. Although only a small number of cases was examined, a correlation was found between *Met*/HGF receptor over-expression and the tendency of HNSCC to result in multiple metastases (Table 2). Statistical analysis using the χ^2 test (with Yates correction) of pN2 and pN3 tumors versus pN0-pN1 tumors confirmed this trend ($P < 0.1$).

Discussion

It is our belief that cancer is a multistep process and the product of the accumulation of many genetic alterations in affected cells. Usually, this process takes place over a span of years and results in the loss of a cell's "social" control. Under physiological conditions, intercellular communication is mediated by growth factors, which initiate a signal that is amplified by intracellular signal effectors. Signalling passes through growth factor receptors spanning the plasma membrane to the nucleus. These receptors are therefore a check-point for cell proliferation and differentiation. Alteration of the genes stimulating or suppressing cell growth and differentiation leads to the emergence of a neoplastic cell clone. These genes (or oncogenes) encode proteins directly related to pathogenesis of the disease, with these proteins serving as the most useful tumor markers.

The current practice of using molecular markers has helped not only in understanding the molecular changes related to the basic biology of human tumors, but also in decisions related to clinical management. Cancer management is now developing into four main branches: (1) prevention; (2) early detection; (3) improvement in the accuracy of diagnosis; (4) new therapeutic strategies adjuvant to conventional therapies. The use of oncogene proteins as molecular markers in studying each of these steps will lead to improved ways of treating disease.

The study of oncogenes in HNSCC may provide insight as to what determines growth and progression of tumors. Genetic studies have shown that certain chromosomal abnormalities recurred in HNSCC [1, 3, 12, 16]. Abnormalities of specific oncogenes have also been reported, including over-expression of growth factors and growth factor receptors [15, 24]. We investigated the role of a growth factor receptor encoded by the *MET* oncogene in

HNSCC and their metastases, since this receptor has been previously related to progression towards metastasis in the pathogenesis of human cancer. In vitro experiments showed that the *Met* receptor and its ligand HGF mediate the ability of transformed cells to invade reconstituted basement membranes. Experimental models showed that activation of the receptor confers to neoplastic cells the ability to metastasize. The *Met*/HGF receptor is over-expressed in human cancer of a specific histotype and is amplified in hematogenous metastases.

Our findings show that the *Met*/HGF receptor is expressed in HNSCC or can be over-expressed at a high level in a few cases and this expression is constantly maintained or increased in synchronous metastases. Two metachronous metastases also showed over-expression. In our series, *Met*/HGF receptor over-expression was found in tumors classified as N2 or N3. This is intriguing, as N staging of HNSCC not only can be correlated with patient outcome [25], but also provide criteria for planning treatment [17]. Follow-up of larger series is necessary to establish if this marker and its level of expression might also help in prognostic evaluations.

Whatever the clinical significance of our data might be, the *Met*/HGF receptor can be used as a marker for the presence of metastatic cells in lymph nodes. Histopathological examination of the tissue samples analyzed in our cases of HNSCC consistently paralleled our molecular diagnoses of metastatic lymph nodes. When the receptor was over-expressed in primary tumors, it was invariably detectable in metastatic cells in the nodes studied. In three cases, enlarged lymph nodes were excised at surgery and examined for suspected metastasis. An accurate immunohistochemical and histopathological examination did not detect any tumor cells in the latter nodes or in many other nodes of the same patient. Receptor was also not detected by protein analysis in the same enlarged nodes. This result may help in diagnosis since a high percentage of primary tumors do express the receptor, and this is reflected in lymph nodes positive for metastatic disease.

When used together, the HGF and *Met* receptor can be considered potential molecular targets for new therapeutic approaches. HNSCCs are characterized by such pathological findings as extracapsular invasion, neurotropism and microscopic residual disease. Persistent disease necessitates innovative therapies that intervene at molecular levels. In addition, recurrences of HNSCC are commonly predominantly local or regional, pointing to regional lymph nodes as primary targets for therapy. Treatment strategies now being designed include targeting with monoclonal antibodies [2, 18], somatic gene therapy [5, 19] and local immunotherapy [7, 8, 23] for microscopic residual disease or lymph node micrometastases. Clayman et al. [5] reported that the in vivo introduction of a wild-type *p53* via an adenoviral vector stops cell growth regardless of the endogenous *p53* gene status of tumor cells and induces morphological changes consistent with apoptosis. In vitro studies show that inhibition of tumor growth may also be related to the arrest of the G_1 cell cycle or to the induction of another tumor suppressor gene, such as *WAF1/CIP1*. Another promising approach to HNSCC management comes from the application of mAbs directed against epidermal growth factor receptor (EGFR). In vitro and in vivo studies have shown that these anti-EGFR mAbs inhibit tumor growth inducing terminal differentiation [18]. These antibodies may be of help in the management of tumor control either alone or conjugated with cytotoxic agents.

Present therapeutic approaches for cancer have developed from the expansion of fundamental knowledge of tumor biology rather than from empiric observations. Thus, our findings that the *Met*/HGF receptor marks HNSCC cells metastasizing to lymph nodes might be applied not only to larger diagnostic and prognostic studies, but also for planning novel therapeutic strategies.

Acknowledgements We thank Rita Callipo and Giovanna Petruccelli for their technical assistance and Elaine Wright and Antonella Cignetto for secretarial help. This paper was supported by grants from the Italian Consiglio Nazionale delle Ricerche (C.N.R., PF ACRO) to M.F. Di Renzo and G. Cortesina.

References

1. Akervall JA, Yuesheng J, Wennerberg JP, Zatterstrom UK, Kjellen E, Mertens F, Willen R, Mandahl N, Heim S, Mitelman F (1995) Chromosomal abnormalities involving 11q13 are associated with poor prognosis in patients with squamous cell carcinoma in the head and neck. Cancer 76: 853–859
2. Brakenhoff RH, Van Gog FB, Looney JE, Van Walsum M, Snow GB, Van Dongen GA (1995) Construction and characterization of the chimeric monoclonal antibody E48 for therapy of head and neck cancer. Cancer Immunol Immunother 40: 191–200
3. Brzoska PM, Levin NA, Fu KK, Kaplan MJ, Singer MI, Gray JW, Christman MF (1995) Frequent novel DNA copy number increase in squamous cell head and neck tumours. Cancer Res 55: 3055–3059
4. Bussolino F, Di Renzo MF, Ziche M, Bocchietto E, Olivero M, Naldini L, Gaudino G, Tamagnone L, Coffer A, Comoglio PM (1992) Hepatocyte growth factor is a potent angiogenic factor which stimulates endothelial cell motility and growth. J Cell Biol 119: 629–641
5. Clayman GL, El-Naggar AK, Zhang WW, Taylor DL, Roth JA, Goepfert H, Liu TJ (1995) In vivo molecular therapy with p53 adenovirus for microscopic residual head and neck squamous carcinoma. Cancer Res 55: 1–6
6. Comoglio PM (1993) Structure, biosynthesis and biochemical properties of the HGF receptor in normal and malignant cells. Hepatocyte growth factor-scatter factor (HGF-SF) and the c-Met receptor. Birkhauser, Basel, pp 131–165
7. Cortesina G, De Stefani A, Giovarelli M, Barioglio MG, Cavallo GP, Forni G (1988) Treatment of recurrent squamous cell carcinoma of the head and neck with low doses of Interleukin-2 injected perilymphatically. Cancer 62: 2462–2465
8. Cortesina G, De Stefani A, Galeazzi E, Cavallo GP, Badellino F, Margarino G, Jemma C, Forni G (1994) Temporary regression of recurrent squamous cell carcinoma of the head and neck is achieved with low but not with a high dose of recombinant Interleukin-2 injected perilymphatically. Br J Cancer 69: 572–576
9. Di Renzo MF, Narsimhan RP, Olivero M, Bretti S, Giordano S, Medico E, Gaglia P, Zara P, Comoglio PM (1991) Expression of the *Met*/HGF receptor in normal and neoplastic human tissues. Oncogene 6: 1997–2003

10. Di Renzo MF, Olivero M, Ferro S, Prat M, Bongarzone I, Pilotti S, Belfiore A, Costantino A, Vigneri R, Pierotti M (1992) Overexpression of the c-*MET*/HGF receptor gene in human thyroid carcinomas. Oncogene 7: 2549–2553
11. Di Renzo MF, Olivero M, Giacomini A, Porte H, Chastre E, Mirossay L, Nordlinger B, Bretti S, Bottardi S, Giordano S, Plebani M, Gespach C, Comoglio PM (1995) Overexpression and amplification of the *Met*/HGF receptor gene during the progression of colorectal cancer. Clin Cancer Res 1: 147–154
12. El-Naggar AK, Hurr K, Batsakis JG, Luna M, Goepfert H, Huff V (1995) Sequential loss of heterozygosity at microsatellite motifs in preinvasive and invasive head and neck squamous carcinoma. Cancer Res 55: 2656–2659
13. Giordano S, Ponzetto C, Di Renzo MF, Cooper CS, Comoglio PM (1989) Tyrosine kinase receptor indistinguishable from the c-met protein. Nature 339: 155–156
14. Goldberg ID, Rosen EM (eds) (1993) Hepatocyte growth factor-scatter factor (HGF-SF) and the c-*Met* receptor. Birkhäuser, Basel
15. Irish JC, Bernstein A (1993) Oncogenes in head and neck cancer. Laryngoscope 103: 42–52
16. Meredith SD, Levine PA, Burns JA, Gaffey MJ, Boyd JC, Weiss LM, Erickson NL, Williams ME (1995) Chromosome 11q13 amplification in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 121: 790–794
17. Million RR, Cassisi NJ, Mancuso AA, Stringer SP, Mendenhall WM, Parsons JT (1994) Management of the neck for SCC. Management of head and neck cancer. A multidisciplinary approach. Lippincott, Philadelphia, pp 75–142
18. Modjtahedi H, Eccles S, Sandle J, Box G, Titley J, Dean C (1994) Differentiation or immune destruction: two pathways for therapy of squamous cell carcinomas with antibodies to the epidermal growth factor receptor. Cancer Res 54: 1695–1701
19. O'Malley BW, Chen SH, Schwartz MR, Woo SL (1995) Adenovirus-mediated gene therapy for human head and neck squamous cell cancer in a nude mouse model. Cancer Res 55: 1080–1085
20. Park M, Dean M, Kaul K, Braun MJ, Gonda MA, Vande Woude G (1987) Sequence of *MET* protooncogene cDNA has features characteristic of the tyrosine kinase family of growth-factor receptors. Proc Natl Acad Sci USA 84: 6379–6383
21. Prat M, Crepaldi T, Gandino L, Giordano S, Longati P, Comoglio PM (1991) C-terminal truncated forms of *Met*, the hepatocyte growth factor receptor. Mol Cell Biol 11: 5954–5962
22. Prat M, Narsimhan RP, Crepaldi T, Nicotra MR, Natali PG, Comoglio PM (1991) The receptor encoded by the human c-*MET* oncogene is expressed in hepatocytes, epithelial cells and solid tumors. Int J Cancer 49: 323–328
23. Rosenberg SA (1988) Immunotherapy of cancer using Interleukin-2. Immunol Today 9: 58–64
24. Schantz SP (1994) Head and neck oncology reasearch. Curr Opin Oncol 6: 265–271
25. Suen JY (1993) Cancer of the neck. Cancer of the head and neck. Churchill Livingstone, London, pp 221–254
26. Weidner KM, Sachs M, Birchmeier W (1993) The *Met* receptor tyrosine kinase transduces motility, proliferation, and morphogenic signals of scatter factor/hepatocyte growth factor in epithelial cells. J Cell Biol 121: 145–154

```
HILIGHT set on as ''
? b 340
       03apr07 10:40:25 User231882 Session D1799.2
            $0.00    0.115 DialUnits File410
     $0.00  Estimated cost File410
     $0.03  TELNET
     $0.03  Estimated cost this search
     $0.03  Estimated total session cost   0.368 DialUnits

File 340:CLAIMS(R)/US Patent 1950-07/Mar 29
       (c) 2007 IFI/CLAIMS(R)
*File 340: The 2006 reload is online as of December 1, 2006.
IPCR/8 is available.

      Set  Items  Description
      ---  -----  -----------
? s gadolinium
      S1    3552  GADOLINIUM
? s mri
      S2    3988  MRI
? s s1 and s2
            3552  S1
            3988  S2
      S3     219  S1 AND S2
? s dtpa
      S4     821  DTPA
? s s3 and s4
             219  S3
             821  S4
      S5      52  S3 AND S4
? s antibod?
      S6   53597  ANTIBOD?
? s s5 and s6
              52  S5
           53597  S6
      S7      12  S5 AND S6
? s s7 and py<2001
              12  S7
         3483355  PY<2001
      S8       0  S7 AND PY<2001
? s dtpa or (diethylenetriaminepentaacetic)
             821  DTPA
             676  DIETHYLENETRIAMINEPENTAACETIC
      S9    1309  DTPA OR (DIETHYLENETRIAMINEPENTAACETIC)
? s s3 and s9
             219  S3
            1309  S9
     S10      60  S3 AND S9
? s s10 and s6
              60  S10
           53597  S6
     S11      14  S10 AND S6
? s s11 not s12
>>>"S12" does not exist
              14  S11
               0  S12
     S12      14  S11 NOT S12
? s s11 not s7
              14  S11
              12  S7
     S13       2  S11 NOT S7
? t s13/3,k,ab/1-2
```

13/3,K,AB/1
DIALOG(R)File 340:CLAIMS(R)/US Patent

Dialog Acc No: 10551733 IFI Acc No: 2004-0058951
IFI Publication Control No: 2004-0058951 IFI Chemical Acc No: 2004-0017482
Document Type: C
INTEGRIN TARGETED IMAGING AGENTS; EMULSION CONTAINING NANOPARTICLES IN PERFLUOROHYDROCARBON SOLVENT; ANTIINFLAMMATORY AGENTS; ANTITUMOR AGENTS
Inventors: Harris Tom (US); Lanza Gregory M (US); Wickline Samuel A (US)
Assignee: Unassigned Or Assigned To Individual
Assignee Code: 68000
Probable Assignee (A1): Barnes Jewish Hosptial; Bristol Myers Imaging Inc
Attorney, Agent or Firm: Kate H. Murashige Morrison & Foerster LLP, Suite 500, 3811 Valley Centre Drive, San Diego, CA, 92130-2332, US
Publication (No,Kind,Date), Applic (No,Date):
US 20040058951 A1 20040325 US 2003351463 20030124
Priority Applic(No,Date): US 2003351463 20030124
Provisional Applic(No,Date): US 60-351390 20020124

Abstract: Emulsions preferably of nanoparticles formed from high boiling liquid perfluorochemical substances, said particles coated with a lipid/surfactant coating are made specific to regions of activated endothelial cells by coupling said nanoparticles to a ligand specific for alpha v beta 3 integrin, other than an ***antibody*** . The nanoparticles may further include biologically active agents, radionuclides, or other imaging agents.

Abstract: ...said nanoparticles to a ligand specific for alpha v beta 3 integrin, other than an ***antibody*** . The nanoparticles may further include biologically active agents, radionuclides, or other imaging agents.

Exemplary Claim:
...alpha v beta 3 integrin, with the proviso that said ligand is other than an ***antibody*** or fragment thereof.
Non-exemplary Claims:
...method of claim 6, wherein said nanoparticles further include at least one magnetic resonance imaging (***MRI***) contrast agent...

...9. The method of claim 8, wherein said ***MRI*** contrast agent is a chelated paramagnetic ion...

...10. The method of claim 9, wherein said chelating agent comprises diethylenetriaminepentaacetic acid or 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid and the paramagnetic ion is ***gadolinium*** ion...
...for alpha v beta 3, with the proviso that said ligand is other than an ***antibody*** or fragment thereof...

...composition of claim 27, wherein said nanoparticles further include at least one magnetic resonance imaging (***MRI***) contrast agent...

...30. The composition of claim 29, wherein said ***MRI*** contrast agent is a chelated paramagnetic ion...

13/3,K,AB/2
DIALOG(R)File 340:CLAIMS(R)/US Patent

Dialog Acc No: 10342453 IFI Acc No: 2003-0086867
IFI Publication Control No: 2003-0086867 IFI Chemical Acc No: 2003-0023568
Document Type: C
BLOOD CLOT-TARGETED NANOPARTICLES; TO DELIVER A RADIONUCLIDE TO A BLOOD CLOT, TO OBTAIN AN ULTRASOUND IMAGE OF A BLOOD CLOT, FOR DIAGNOSIS, THERAPY
Inventors: Lanza Gregory (US); Wickline Samuel A (US)
Assignee: Unassigned Or Assigned To Individual
Assignee Code: 68000
Probable Assignee (A1): Barnes Hospital
Attorney, Agent or Firm: Kate H. Murashige Morrison & Foerster LLP, Suite 500, 3811 Valley Centre Drive, San Diego, CA, 92130-2332, US
Publication (No,Kind,Date), Applic (No,Date):
US 20030086867 A1 20030508 US 2002225024 20020820
Cont.-in-part Pub(No),Applic(No,Date): PENDING US 99404963 19990924
Priority Applic(No,Date): US 2002225024 20020820; US 99404963 19990924

Abstract: Emulsions comprising nanoparticles formed from high boiling perfluorochemical substances, said particles coated with a lipid/surfactant coating are made target-specific by directly coupling said nanoparticles to a targeting ligand. The nanoparticles may further include biologically active agents, radionuclides, and/or other imaging agents.

Non-exemplary Claims:
...composition of claim 1, wherein said nanoparticles further include at least one magnetic resonance imaging (***MRI***) contrast agent...

...5. The composition of claim 4, wherein said ***MRI*** contrast agent is a chelated paramagnetic ion...

...6. The composition of claim 5, wherein said chelating agent is diethylenetriaminepentaacetic acid and the paramagnetic ion is ***gadolinium*** ion...

...11. The composition of claim 1, wherein said targeting ligand is an antibody, a fragment of an antibody, an aptamer, a hormone, peptidomimetic or a receptor ligand...

...12. The composition of claim 11, wherein the targeting ligand is an ***antibody*** or fragment of an ***antibody*** .
...
...13. The composition of claim 12, wherein said ***antibody*** or fragment is humanized
?

NPL___ ADONIS___ MIC___ BioT___Main__NO__Vol NO✓__ NOS___CKCite__Dup__Int M

STIC-ILL 2224 2997 623533

From: Davis, Minh-Tam
Sent: Tuesday, April 03, 2007 12:09 PM
To: STIC-ILL
Subject: REPRINT REQUEST FOR 10/500297

1) Magnetic resonance imaging studies on nude mice grafted with colorectal adenocarcinoma using gadolinium-labeled monoclonal antibody.
Saccavini J C; Curtet C; Bohy J; Tellier C; Bourgoin C; Lhoste J M
Oris Industrie, Gif sur Yvette, France.
Investigative radiology (UNITED STATES) Sep 1988, 23 Suppl 1 pS292-3, ISSN 0020-9996--Print Journal Code: 0045377

COMPLETED

3198365

2) MRI contrast enhancement by Gd-DTPA-monoclonal antibody in 9L glioma rats.
Matsumura A; Shibata Y; Nakagawa K; Nose T
Department of Neurosurgery, University of Tsukuba, Ibaraki, Japan.
Acta neurochirurgica. Supplementum (AUSTRIA) 1994, 60 p356-8, ISSN 0065-1419--Print Journal Code: 0140560

THANK YOU
MINH TAM DAVIS
ART UNIT 1642, REMSEN 3A24, MB 3C18
272-0830

Magnetic Resonance Imaging Studies on Nude Mice Grafted with Colorectal Adenocarcinoma Using Gadolinium-Labeled Monoclonal Antibody

J. C. SACCAVINI,* C. CURTET,† J. BOHY,* C. TELLIER,‡ C. BOURGOIN,* AND J. M. LHOSTE§

Saccavini JC, Curtet C, Bohy J, Tellier C, Bourgoin C, Lhoste JM. Magnetic resonance imaging studies on nude mice grafted with colorectal adenocarcinoma using gadolinium-labeled monoclonal antibody. Invest Radiol 1988;23(Suppl 1):S292–S293.

A monoclonal antibody (Ab) 19.9 specific for colorectal carcinoma was labeled with a high number of gadolinium (Gd) atoms for its potential application as a contrast agent in magnetic resonance imaging (MRI). The DTPA was conjugated to 19.9 Ab via the bicyclic DTPA anhydride method (c.DTPA) using c.DTPA/Ab molar ratios between 5 and 150. The aggregates present in great amount at high c.DTPA/Ab ratios were systematically removed. Then the exact number of DTPA effectively conjugated, the immunoreactivity of the resulting ^{111}In-DTPA-Ab were measured. The number of DTPA conjugated per antibody can be increased 20 to 25 with only a little loss of immunoreactivity. The 19.9 antibody conjugated with 16 and 25 DTPA was labeled with $^{153}GdCl_3$ for pharmacokinetic studies on xenografted nude mice and with nonradioactive gadolinium to measure ex vivo the effect on the relaxation time T1 of the tumor. We found a 15 to 20% decrease of T1 on the tumor. In vivo experiments using a Bruker system and the same animal model showed a difference in the tumor contrast after the injection of 2 mg of Gd-labeled Ab.

SCINTIGRAPHY IS A very sensitive technique. A specific accumulation of a small amount of radiolabeled antibodies (about 10^{-12} radioactive atoms) is sufficient to get an external image of the tumor. Magnetic resonance imaging (MRI) needs at least 10^{-5} paramagnetic atoms in the tumor to get a contrasted image[1]; between the two imaging methods the difference in sensitivity is about 10^7. To succeed in MRI with antibodies, we have to consider the following parameters: to increase the quantity of antibody injected, to choose antibodies that recognize a high number of antigenic sites per tumor cell, and to label each antibody with a high number of gadolinium (Gd) atoms.

This last point, the labeling of antibodies with a high number of gadolinium atoms via bifunctional chelates was considered. The DTPA was conjugated to 19.9 antibody (Ab) specific for colorectal carcinoma via the bicyclic DTPA anhydride (c.DTPA conjugation on antibody immunoreactivity, various c.DTPA/Ab ratios were used. The high c.DTPA/Ab ratios resulted in the formation of aggregates which involve a decrease in antibody immunoreactivity (Table 1). But when the aggregates were removed (5 DTPA) 19.9 Ab and (7.6 DTPA)-19.9 Ab recovered 70% of immunoreactivity. So the major problem in the decrease of the immunoreactivity is the presence of aggregates.

A study with higher c.DTPA/Ab ratios was performed to determine the influence of the number of DTPA conjugated per antibody on the immunoreactivity. For this study, the aggregates were systematically removed by gel filtration, then the exact number of DTPA conjugated per antibody was determined using a titration method based on a competition between In Cl_3 and radioactive carrier free $^{111}InCl_3$.

The immunoreactivity of each antibody labeled with ^{111}In was measured by affinity chromatography and the tumor uptake was measured after injection on xenografted nude mice (Table 2).

Many reports indicate that the number of DTPA conjugated per antibody do not exceed two[3]; but if we take care to remove the aggregates, the number of DTPA conjugated per antibody can be increased 15 to 20 with only a little loss of immunoreactivity.

From *Oris Industrie, Gif sur Yvette, †Inserm U211, Nantes, ‡Uer des Sciences de la Nature, Nantes, and §Institute Curie, Inserum U219, Orsay, France.

Reprint requests: J. C. Saccavini, Oris Industrie, B.P. 21, 91190, Gif sur Yvette, France.

Suppl. 1

1

c.DTPA/Ab Ratios

25
5
10
20

TABLE : Antil

c.DTPA/Ab Ratios

5
50
100
150

Fig. 1. mice with :

TABLE 1. Effect of DTPA Conjugation on Antibody Immunoreactivity

c.DTPA/Ab Ratios	No. of DTPA Conjugated	Aggregates U.V. Determination	Immunoreactivity
2.5	1	-	72%
5	1.8	5%	70%
10	5	10%–15%	64%
20	7.6	15%–20%	53%

TABLE 2. Effect of the Number of DTPA Conjugated per Antibody on Immunoreactivity and Tumor Uptake

c.DTPA/Ab Ratios	No. of DTPA Conjugated	% of Aggregates	Immuno-reactivity	Tumor Uptake Injected dose/g
5	2	< 1%	70%	4.23%
50	16	4%	60%	3.21%
100	25	6%	33%	2.70%
150	50	3%	15%	1.08%

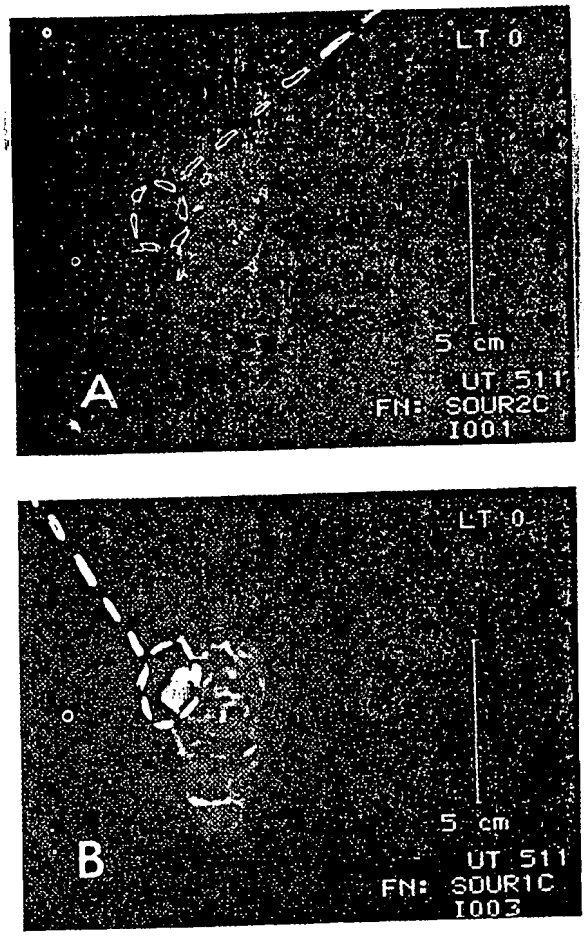

Fig. 1. Tumor area. (A) Nude mice without injection. (B) Nude mice with 2 mg-Injection antibody Gd-DTPA.

TABLE 3. Relaxation Time T1 of Water Proton in Human Tumor Grafted in Nude Mice

	Measure of T1 at 90 MHz
Normal tissue	T1 (ms)
colon	470 ± 45
Human tumor	
HRT 18 + 19.9 Ab	1228 ± 13
HRT 18 + Gd Cl_3	1205 ± 13
HRT 18 + Gd-DTPA	1239 ± 23
HRT 18 + Gd-16DTPA-19.9Ab	1046 ± 29 (↘15%)
	Measure of T1 at 20 MHz
HRT 18	858.5
SW 948	881
HRT 18 + Gd-25DTPA-19.9Ab	756 (↘12%)
SW 948 + Gd-25DTPA-19.9Ab	694 (↘21%)

The 19.9 antibody chelated with 16 DTPA and an irrelevant antibody (antihepatitis A_2C_6) also conjugated with 16 DTPA were labeled with $^{153}Gd\ Cl_3$ with a labeling efficiency of 50%. The pharmacokinetic studies showed a specific 19.9 antibody uptake by the tumor (5.08% for 19.9 vs. 1.92% for A_2C_6), whereas the liver (4.12% vs. 4.52%) and blood (1.65% vs. 1.72%) uptake was the same. The same 19.9 Ab conjugated with 16 and 25 DTPA was labeled with nonradioactive gadolinium with a labeling efficiency of 90%. These antibody solutions were infused into nude mice xenografted with two human colic tumors HRT 18 and SW 948. One day after the injection of 2 mg of antibody solution in 0.3 mL, the tumors were removed and the relaxation time T1 measured at 90 MHz and 20 MHz using the inversion recuperation method. Using the Gd-16 DTPA-19.9 Ab solution and HRT 18 cell line a 15% tumor T1 decrease was observed at 90 MHz, using the Gd-25 DTPA-19.9 Ab solution and Sw 948 cell line a 21% tumor T1 decrease was observed at 20 MHz (Table 3).

MR scanning was performed using a 4.7-T Bruker Instrument. Spin-echo phase sequences (T_R500, T_E30 ms) were used.

The animal was disposed in a Plexiglas box which held the animal rigidly. Transverse and coronal 4-mm sections were performed. Significant differences in the tumor contrast were observed between nude mice injected with 2 mg of gadolinium labeled antibody and nude mice alone or injected with Gd-DTPA (Fig. 1)

References

1. Strich G, Hagan PL, Gerber KM, Slutsky RA. Tissue distribution and magnetic resonance spin lattice relaxation effects on Gd-DTPA. Radiology 1985;154:723.
2. Hnatowich DT, Layne WW, Childs RL. The preparation and labeling of DTPA coupled albumin. Int J Appl Radiat Isot 1982,33:327.
3. Paik CH, Ebbert MA, Murphy PL, et al. Factors influencing DTPA conjugation to antibodies via cyclic DTPA anhydride. J Nucl Med 1983;24:1158.

#25.00

NPL___ ADONIS_✓_ MIC___ BioT___Main__NO__Vol NO__ NOS___CKCite__Dup__Int MC

STIC-ILL

623690

From: Davis, Minh-Tam
Sent: Tuesday, April 03, 2007 12:09 PM
To: STIC-ILL
Subject: REPRINT REQUEST FOR 10/500297

1) Magnetic resonance imaging studies on nude mice grafted with colorectal adenocarcinoma using gadolinium-labeled monoclonal antibody.
Saccavini J C; Curtet C; Bohy J; Tellier C; Bourgoin C; Lhoste J M
Oris Industrie, Gif sur Yvette, France.
Investigative radiology (UNITED STATES) Sep 1988, 23 Suppl 1 pS292-3, ISSN 0020-9996--Print Journal Code: 0045377

2) MRI contrast enhancement by Gd-DTPA-monoclonal antibody in 9L glioma rats.
Matsumura A; Shibata Y; Nakagawa K; Nose T
Department of Neurosurgery, University of Tsukuba, Ibaraki, Japan.
Acta neurochirurgica. Supplementum (AUSTRIA) 1994, 60 p356-8, ISSN 0065-1419--Print Journal Code: 0140560

THANK YOU
MINH TAM DAVIS
ART UNIT 1642, REMSEN 3A24, MB 3C18
272-0830

Article title	MRI Contrast Enhancement by Gd-DTPA-Monoclonal Antibody in 9L Glioma Rats
Article identifier	006514199410096S
Authors	Matsumura_A Shibata_Y Nakagawa_K Nose_T
Journal title	Acta Neurochirurgica, Supplement
ISSN	0065-1419
Publisher	Springer Wien
Year of publication	1994
Volume	-
Issue	S60
Supplement	1
Page range	356-358
Number of pages	3
User name	Adonis
Cost centre	
PCC	$25.00
Date and time	Wednesday, April 04, 2007 9:58:27 AM

Acta Neurochir (1994) [Suppl] 60: 356–358

MRI Contrast Enhancement by Gd-DTPA-Monoclonal Antibody in 9L Glioma Rats

A. Matsumura, Y. Shibata, K. Nakagawa, and T. Nose

Department of Neurosurgery, Institute of Clinical Medical Science, University of Tsukuba, Tsukuba, Ibaraki, Japan

Summary

To achieve a tissue-specific enhancement in diagnosis of brain tumor, a magnetic resonance imaging (MRI) study was performed using conjugate of Gd-DTPA and monoclonal antibody (MoAb) against 9L glioma cells. Fisher 344 strain rats were used for this study. MoAb against 9L glioma cells was conjugated with Gd-DTPA according to the method of Hnatowich *et al.* (1983) and used for the MRI study. The gadolinium (Gd) concentration in the Gd-MoAb injected to the rats was 0.01–0.03 mmol/kg. The enhancement effect increased gradually and persisted for 24 hours after the injection. This was longer than Gd-DTPA, which showed a peak of enhancement effect within 30 minutes after injection and was washed out within 120 min. This result was compatible with scintigraphy studies using ^{125}I labeled anti 9L monoclonal antibody, in which the accumulation of the ^{125}I antibody increased at 24, 48 and 72 hours after the injection. By using tumor-specific contrast agents such as Gd-MoAb, it may be possible to differentiate among tumor, perifocal edema and radiation injury.

Keywords: Magnetic resonance imaging; brain tumor; monoclonal antibody; gadolinium.

Introduction

Gadopentate dimegulumine (Gd-DTPA) is widely used as a contrast agent for magnetic resonance imaging (MRI). Gd-DTPA is useful in delineating the margin of the tumor on T1-weighted images.

However, it is known that Gd-DTPA crosses the damaged blood-brain barrier (BBB), and thus infarction area and radiation necrosis are also enhanced by Gd-DTPA. Therefore, the developement of tissue-specific contrast agents deserves considerable attention due to their specificity to the tumor. The use of monoclonal antibody as an MRI contrast agent in subcutaneous tumor in nude mice has been successful[1]. In the present study, we demonstrate the tumor-specific enhancement of intracerebrally transplanted 9L glioma tumor in Fisher 344 rats and discuss the usefullness and limitations of this contrast agent.

Materials and Methods

Monoclonal antibody (MoAb) against 9L glioma cells was produced by using hybridoma of Balb/c mouse spleen cells and P3U1 myeloma cells.

For the scintigraphy study, ^{125}I labeled monoclonal antibody to 9L was used. Nonspecific IgG was also labeled by ^{125}I and served as a control. Scintigraphy was performed in Fisher rats with subcutaneous 9L tumor.

The Gd-MoAb was conjugated according to the method of Hnatowich *et al.* (1983)[2]. DTPA anhydride and 9L monoclonal antibody were first conjugated, and then, in order to remove the excess DTPA, gel fitration through a Sephadex G-50 was performed. Next, Gd-acetate was added to label DTPA-MoAb with Gd. The final molar ratio was 1 : 1.

For the experimental brain tumor model, Fisher 344 strain rats were used. 1×10^4/10 µl 9L glioma cells were innoculated into the brain parenchyma at a depth of 5 mm from the surface through a burr hole near the coronal suture using a stereotactic manipulator with a 27G fine needle.

MRI was performed 14 days after the innoculation under general anesthesia by pentobarbital with spontaneous breathing. MRI was performed by a BMT24/40 superconducting MRI system (Bruker Co., Ltd., 2.4T) by using spin echo images for T1-weighted (Tr/Te = 512/28) and T2-weighted (Tr/Te = 2040/80) images. The enhancement effect of Gd-MoAb was compared with that of Gd-DTPA.

After MRI analysis, the rats were sacrificed using an overdose of pentobarbital. Tumor, brain, skin, liver, kidney and serum were sampled and immediately kept in a deep freezer until being used for the analysis. Gd concentrations in the tissue were measured by an ICP-analyzer (Seiko Electric Co., Ltd., SPS 1200A).

Results

^{125}I MoAb Scintigraphy Study

After injection of ^{125}I-MoAb, the rats were serially evaluated by scintigraphy. As shown in Fig. 1, ^{125}I-

MoAb to 9 L cells (left side) accumulated in the tumor (black arrows) and in the liver after 48 hours, while nonspecific IgG (right side) showed no accumulation in the tumor (white arrows).

MRI Study

The gadolinium concentration in the Gd-MoAb injected to the rats was .0.01–0.03 mmol/kg. The enhancement effect of Gd-MoAb increased gradually and persisted for 24 hours after the injection (Fig. 2), although the change in intensity was rather weak compared to that of Gd-DTPA. Gd-DTPA injected at 0.1 mmol/kg showed a peak of enhancement effect in the tumor within 30 minutes after injection, and there was almost no enhancement effect after 120 minutes.

Tissue Gd Concentration

The tissue concentration of Gd in the Gd-DTPA group rapidly increased in the serum, kidney and tumor, while there was only very low accumulation in the normal brain. The peak concentration of gadolinium in the tumor was 18 ppm. The clearance of Gd-DTPA from all tissues was fairly rapid. Unlike Gd-DTPA, Gd-MoAb did not accumulate in the kidney, but mainly in the liver. The gadolinium concentration in the tumor was below 0.03 μg/g tissue throughout the entire time course in the majority of cases. The accumulation of gadolinium in the liver slowly increased after injection and reached about 40 ppm after 48 hours.

Discussion

Monoclonal antibody to 9L glioma cells exhibited a specific binding activity to intracerebrally transplanted experimental tumor cells evaluated by both scintigraphy and MRI.

The enhancement effect of Gd-MoAb on MRI continued for 24 hours after injection. This result was compatible with a scintigraphy using ^{125}I labeled anti-9L monoclonal antibody study in which accumulation of the ^{125}I-MoAb in the tumor relatively increased at 24, 48 and 72 hours after the injection. The results indicate tissue-specific binding activity and this may be useful in differential diagnosis of various types of tumor and/or damaged blood-brain barrier (e.g. cerebral infarction and radiation necrosis).

The increase of signal intensity of MRI was rather weaken with Gd-MoAb than with Gd-DTPA. This is thought to be due to the amount of Gd given to the rats.

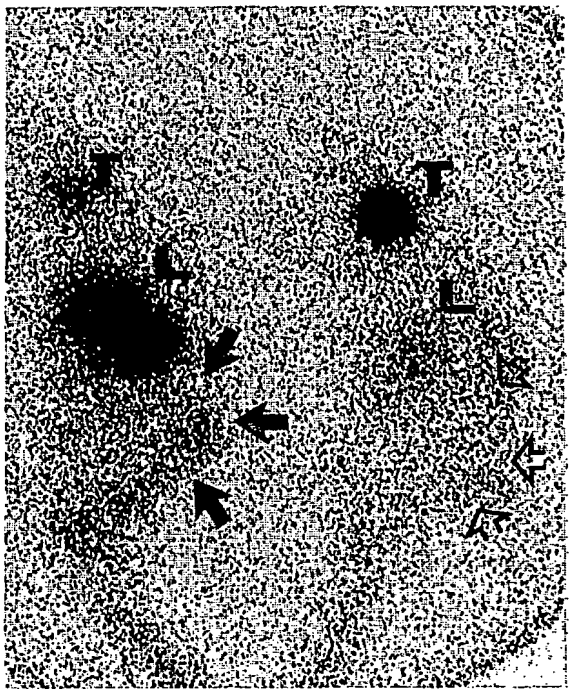

Fig. 1. ^{125}I scintigraphy of 9L monoclonal antibody (left side) and non specific IgG (right side) in 9L tumor-bearing rats. Note the accumulation in the tumor (*T*), with monoclonal antibody (black arrows), but no accumulation with IgG (white arrows). *L* liver, *T* thyroid

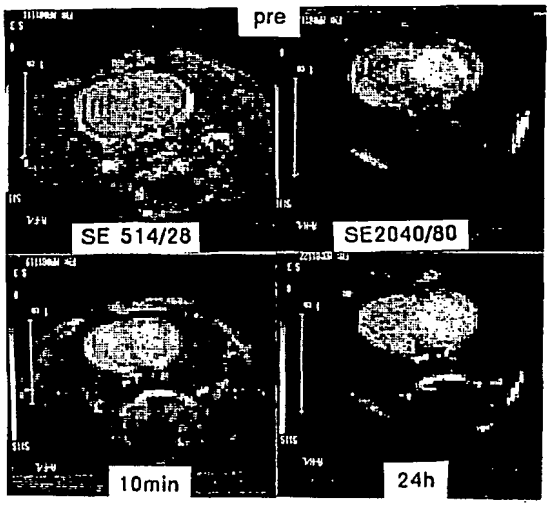

Fig. 2. MRI showing the 9L tumor. Upper row showing pre-contrast T1 image (left) and T2 image (right). After injection of Gd-MoAb, the tumor is enhanced aftr 10 min, and also after 24 hours

Gd-DTPA is used at 0.1 mmol/kg in clinical settings, and so this concentration was used in this study. Gd-MoAb could be administrated at only 0.01–0.03 mmol/kg in the present study due to the technical limitations

of injecting a large amount of Gd-MoAb to the rats. Another reason for the faint and delayed enhancement effect is the high molecular weight of Gd-MoAb. While the molecular weight of Gd-DTPA is only 743, that of Gd-MoAb is about 160,000, which is 200 times that of Gd-DTPA, and therefore its uptake in the tumor is limited.

Gd-MoAb enhancement has been succesful in various subcutaneous tumors using nude mice[1]. Another study using normal hamsters and CEA failed to demonstrate a contrast enhancement[4]. As demonstrated from our data, for in vivo use of Gd-MoAb in subjects with normal immunological systems, the accumulation of Gd-MoAb in the liver makes it difficult to achieve sufficient accumulation of Gd-MoAb in the tumor itself. To overcome this problem, several improvements has to be developed. The intra-arterial administration of monoclonal antibody[3], the use of only Fab fragments in order to decrease the molecular weight[5], and the use of polylysine for more effective labeling of gadolinium[5] are all under investigation. If these improvements are succesful, tumor-specific contrast agents such as Gd-MoAb may become useful in the differential diagnosis of various types of tumor and also in the delineation of perifocal edema and/or radiation injury.

Acknowledgement

This work was supported by University of Tsukuba Research Projects. The authors express their gratitude to Schering Japan Co., Ltd. for their assistance.

References

1. Göhr-Rosenthal S, Schmitt-Willich, Ebert W, Gries H, Vogler II, Weinmann HJ, Conrad J (1991) An immunoselective contrast medium for MRI: detection of colorectal tumor transplants in mice with a Gd-labeled monoclonal antibody. Abstract of 10th SMRM, p 356
2. Hnatowich DJ, Childs RL, Lanteigne D, Najafi A (1983) The preparation of DTPA-coupled antibodies radiolabeled with metallic radionuclides: an improved method. J Immunol Meth 65: 147–157
3. Ingvar C, Norrgren K, Strand SE, Brodin T, Jönsson PE, Sjögren HO (1991) Tumor uptake of monoclonal antibody after regional intraarterial injection. Acta Oncol 30: 65–69
4. Unger EC, Totty WG, Neufeld DM, Otsuka FL, Murphy WA, Welch MS, Connet JM, Philpott GW (1985) Magnetic resonance imaging using gadolinium-labeled monoclonal antibody. Invest Radiol 20: 693–700
5. Wang TST, Fawwaz RA, Alderson PO (1992) Reduced hepatic accumulation of radiolabeled monoclonal antibodies with indium-111-thioether-poly-L-lysine-DTPA-monoclonal antibody-TP41.2F (ab')2. J Nucl Med 33: 570–574

Correspondence: A. Matsumura, M.D., Department of Neurosurgery, Institute of Clinical Medical Science, University of Tsukuba, Ibaraki 305, Japan.